Studies on the control of digestive enzyme secretion and post-ecdysial tanning in Tenebrio molitor L.

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STUDIES ON THE CONTROL OF DIGESTIVE ENZYME SECRETION AND POST-ECDYSIAL TANNING IN *TENEBRIO MOLITOR* L.

Submitted by Yahya Mohammed Abboud

for the degree of Ph.D. of the

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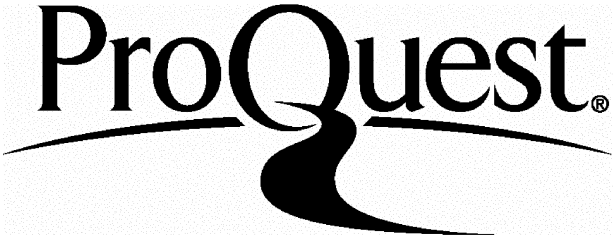

CONTENTS

SUMMARY

Using the adult *Calliphora* bioassay the tanning hormone, bursicon, has been found in the blood of pupal and adult *Tenebrio* only at the time of ecdysis. Contrary to other workers, no evidence could be found for the presence of the hormone in the haemolymph during pharate adult development, before ecdysis begins. When newly ecdysed pupae were ligated about the neck, post-ecdysial tanning of the adult cuticle was almost completely prevented. This failure to tan was not due to a lack of bursicon as the hormone was released normally in the ligated insects at the time of ecdysis. This suggests that a pre-ecdysial signal may be required for the development of epidermal competence to respond to bursicon. Other events were found with similar critical periods for the head to that for bursicon responsiveness e.g. pre-ecdysial plasticization of the elytral cuticle. The results are discussed in terms of a single endocrine effector. However, attempts to demonstrate the presence of a factor in blood and nerve cord of pharate adults that promotes competence to respond to bursicon failed.

A study has been made on the control of digestive enzyme secretion in young adult *Tenebrio*. A spontaneous increase in midgut α-glucosidase activity was observed in the first few days of adult life. This was prevented by neck ligation >1 day before ecdysis. Ligation experiments revealed a critical period for the head in post-ecdysial midgut enzyme activity

during the 24 h prior to ecdysis comparable to that for competence to respond to bursicon. Transfusing neck-ligated insects with pharate adult blood promoted a significant increase in midgut α-glucosidase activity suggesting that a hormone is involved.

A fine structural study has been made of the enterocytes in the midguts of insects starved or fed from emergence for 4 days. The cytological features of the enterocytes are related to the processes of enzyme secretion and peritrophic membrane formation. Cytoplasmic extrusions were only found in the fed insect and it is concluded that they are the result of cell breakdown associated with a high rate of enterocyte turn-over. Although 3 "types" of enterocyte are described, it is suggested that they may simply reflect different stages in the ageing of a single population of cells.

ACKNOWLEDGEMENTS

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I would like to acknowledge the support of a British Council Grant.

CHAPTER 1

General Materials and Methods

1. Maintenance of the insects

Mealworms, *Tenebrio molitor* L., were purchased from suppliers as mature larvae. They were fed on bran, carrots and potatoes. The insects were maintained in an incubator (Gallenkamp) at 25°C and approx. 50% R.H. under a 12 h light: 12 h dark photoperiodic regime. Newly moulted pupae (either 1-3 h or 6 h ± 6 h old) were collected and placed in small tubs.

2. Staging of adult development

Adult development was staged as days after pupation, except for pharate adults nearing the time of ecdysis in which case the system outlined in Table 1 was used. This is based on observations made on over 1000 individual pupae. In those instances where insects ecdysed prior to use, or when the experiment was non-destructive, staging was confirmed by noting the time of onset of ecdysial behaviour. The efficiency of the scoring system was confirmed by the experiment described in Table 2.

3. Application of ligatures and treatment of experimental insects

Non-anaesthetised insects of appropriate stage were ligated with cotton thread (J. and P. Coates, No. 36) around the arthrodial membrane between head and thorax or thorax and abdomen. Experimental insects were kept in groups in petri dishes in the incubator with the stock insects (see 1 above)

4. Preparation of blood and tissue samples

Blood samples were taken from *Tenebrio* by amputating a metathoracic leg; in some cases very gentle pressure was applied to facilitate bleeding. The haemolymph was collected in 5 µl Drummond microcaps, and stored at -20^{o}C until required. For tissue samples, insects were dissected under Ephrussi and Beadle's saline (E/B 7.5 g NaCl, 0.35 g KCl, 0.21 g $CaCl_2$)the central nerve cord (CNS) removed, then briefly blotted and stored frozen. When needed for bioassay, pools of 20 tissues were suspended in 160 µl saline solution, sonicated for 2 min (MSE Soniprep 150) and centrifuged briefly to remove cellular debris (Eppendorf 5412 micro centrifuge).

5. Injection of tissue samples

Injections (4 µl) of blood or tissue extracts were made

dorso-ventrally into the abdomen of non-anaesthetised insects between the 4th and 5th segments using a calibrated glass needle attached to a mouthpiece by a rubber tube. Controls were either pricked with the needle or injected with 4 µl of E/B as stated in the text.

6. Statistical analysis of results

Statistical comparisons of data were performed using Student's t test as described by Snedecor and Cochran (1967). Where appropriate, reference was made to the statistical tables of Fischer and Yates (1963). Values of $P \leq 0.05$ were taken as being significant.

Table 1. Staging of adult development in *Tenebrio molitor*.

Approx. hours before adult ecdysis	Characteristics
24 (27-21)	tarsi show *definite* stripes (i.e. tarsal joints tanned light brown), femur-tibia joints tanned light brown.
18 (21-15)	as for 24 h but tanned regions are darker in appearance, "claws" of the tarsi show some movement.
18 "late"*	tarsal segments and tibia start to tan (golden brown in colour), in addition the joints continue to darken.
12 (14-9.5)	as for 18 h "late", moulting fluid absorption starts from some tarsi.
12 "late"* (10-8)	reabsorption of moulting fluid from the legs is complete.
6 - start of ecdysis	old pupal cuticle becomes progressively softer to the touch, appendages and head can be twisted without severing arthrodial membranes, rate of abdominal flexing increases sharply (see Fig. 12, Chapter 2).

* Not as precise as the other stages (see Table 2).

Table 2. Validation of the scoring system employed to stage adult development in *Tenebrio molitor*.

Groups of pharate adults were scored using the system shown in Table 1 and then placed in an incubator at 25°C to complete development. The time at which the insects ecdysed to adult was noted and compared with the "assessed age".

Stage of adult development in hours before ecdysis ("assessed age")	Time of ecdysis of the group of insects after initial scoring, in hours	Number of insects
24	24 ± 3	55
18	18 ± 3	55
12	12 ± 2.5	45
6	6 ± 2	45
3	3 ± 0.5	55

CHAPTER 2

Bursicon in the mealworm, *Tenebrio molitor* and its role in the control of postecdysial tanning

Introduction

The final sequence of events during an insect moult is termed ecdysis, it consists of splitting and shedding the remains of the old cuticle, expansion of the new cuticle, and hardening (and often darkening) of the newly expanded cuticle. In saturniid and sphingid moths such as *Manduca sexta* adult eclosion (= ecdysis) is triggered by the blood-borne eclosion hormone (Truman and Riddiford, 1970). Recent evidence suggests that this hormone may have a controlling influence in ecdysis in many other insects (Truman *et al.*, 1981). Cuticular hardening (= tanning or sclerotization) is also under endocrine control. However, in this instance there is no doubt that the hormone involved, bursicon, occurs widely in insects. One prominent exception is the specialised case of the hardening of the blowfly puparium which is controlled by the puparium tanning factor PTF (Swasubramanian *et al.*, 1974; Fraenkel *et al.*, 1972). Cottrell (1964) and Reynolds (1980) have reviewed the process of ecdysis generally. Other reviews include a consideration of bursicon e.g. Goldsworthy and Mordue (1974), Neville (1975), Riddiford and Truman, (1978), while specialised reviews on bursicon have been published by Seligman (1980) and Reynolds (1983).

Fraenkel (1936) found that newly emerged blowflies delayed expansion of the body and hardening of the cuticle for many hours when they were constrained in an environment of sawdust. However, it wasn't until 26 years later that, following on from Fraenkel's initial observations, Cottrell (1962a,b) and Fraenkel and Hsiao (1962) showed that the hardening and darkening of the cuticle of newly emerged flies was due to the action of a new hormone, which Fraenkel and Hsaio (1965) termed bursicon. Primary evidence for its existence was provided by the effects of timed decapitation (Cottrell, 1962a) and neck ligation (Fraenkel and Hsiao, 1962) experiments. These procedures prevented darkening of the body if performed within a few minutes of adult emergence but not if they were delayed. Blood from newly moulted flies caused tanning when injected into flies which had been operated on prior to the critical period. Since the chemistry of the blood-borne factor (a peptide) and its properties were inconsistent with its being either a tanning precursor (Fraenkel and Hsiao, 1965) or one of the major developmental hormones viz. PTTH, ecdysone and JH (Cottrell, 1962b), the factor was designated a hormone in its own right.

Over the last 20 years it has been shown that bursicon occurs widely amongst insects. Using either the ligated newly-emerged blowfly as a bioassay or a similar conspecific preparation, the hormone has been demonstrated in Coleoptera, Diptera, Lepidoptera and Orthoptera (see Reynolds, 1983).

Considerable variation exists amongst species regarding the site of release. In blowflies, though large amounts of bursicon activity are present in medial neurosecretory cells (mnc) of the brain, and isolation or removal of the head prevents release, considerable more hormone is present in the posterior half of the fused thoracico-abdominal ganglion, which is considered to be the site of release (Fraenkel and Hsiao, 1963, 1965; Reynolds, 1976). Reported sites of release in other insects are: the last abdominal ganglion in *Periplaneta americana* (Mills, 1965; Mills *et al* ., 1965) and *Locusta migratoria* (Vincent, 1971, 1972), thoracic ganglion in *Leucophaea maderae* (Srivastava and Hopkins, 1975), abdominal perivisceral organs in *Manduca sexta* (Truman, 1973), neurosecretory axon terminals of nerves posterior to the metathoracic ganglion in 1st instar larvae of *Schistocerca gregaria* (Padgham, 1976).

The pattern of release of the hormone is broadly similar in all of the above insects. Namely that the hormone is released into the blood in large amounts during or some time after ecdysis. Since at the peak of activity, blood from *Sarcophaga bullata* can be diluted some 30 fold before injection into a ligated test insects and still give a maximum response (Fraenkel and Hsiao, 1965), it may be supposed that the quantity of hormone released is unimportant so long as a threshold "trigger" level is achieved. The ½-life of the hormone in the blood is characteristically short i.e. from

a few minutes (*Manduca sexta*, Reynolds *et al.*, 1979; *Lucilia cuprina*, Seligman and Doy, 1973) to a few hours (*Periplaneta americana*, Mills, 1966).

Initiation of release in blowflies and adult *Manduca sexta* is separated in time from the physical act of shedding the exuvia. In *Sarcophaga bullata* bursicon is only released after the fly emerges onto a clear surface and stops digging (Fraenkel and Hsiao, 1965). Air swallowing is initiated at this time but is neither a cause nor a consequence of bursicon release. Environmental cues are also important in bringing about the release of bursicon in *M. sexta*, where tanning and wing expansion are delayed until the newly ecdysed adult is unconstrained and has a suitable perch (Reynolds *et al.*, 1979). In contrast, the sensory input required for the release of bursicon in cockroaches (Srivastava and Hopkins, 1975) and 1st instar larvae of *Schistocerca gregaria* (Padgham. 1976) comes from the movement of the old cuticle over the surface of the new during ecdysis.

The bioassays for bursicon activity, particularly the adult blowfly test, do not evaluate hardness but darkening (= melanization). It is generally assumed that hardening and darkening normally occur together and therefore that darkening is a valid measure of the activity of the "tanning" hormone. This seems a reasonable assumption because dihydroxyphenylalanine (DOPA), which is a precursor not only

for the production of tanning agents but also of melanin, is the end product of the rate limiting, bursicon controlled, reaction viz. hydroxylation of tyrosine.

There is no doubt that bursicon eventually does cause sclerotization of blowfly cuticle (Reynolds, 1976). However, this is preceded by a period of general cuticular plasticization which is also bursicon-mediated (Reynolds, 1976). The object of this phenomenon is apparently to facilitate expansion of the new cuticle during air swallowing. Bursicon-mediated cuticular plasticization also occurs postecdysially in adult *M. sexta* (Reynolds, 1977). In the latter case plasticization starts prior to ecdysis and is initiated by the eclosion hormone (Reynolds and Truman, 1982).

The involvement of bursicon in ecdysis is not confined to the control of melanization, sclerotization and plasticization. It appears that bursicon is essential for normal post-ecdysial endocuticle formation in *S. bullata* (Fogal and Fraenkel, 1969) and *L. migratoria* (Vincent, 1971). Additionally bursicon is known to be responsible for the programmed cell death of the wing epidermis in newly emerged *Lucilia cuprina* (Seligman and Doy, 1972). The abaxial and adaxial cuticular surfaces of the newly emerged fly wing are each attached to an epidermal layer. The cells of the two layers are joined together by muscle insertion-like

processes. The last named prevent "ballooning" of the wings during the period of increased blood pressure caused by air swallowing. The importance of these structures is seen when blood pressure is artificially increased after the expansion phase, when the cellular processes between the two surfaces of the wing have broken down. Under these circumstances the wings become distorted and distended with blood (Cottrell, 1962c). Seligman *et al.* (1975) showed that when bursicon release was prevented by neck ligation, the wing epidermis did not break-down and cellular fragments did not appear in the blood. Injection of blood from newly moulted flies into neck ligated insects caused cuticular tanning and appearance of cellular fragments.

Two other phenomena have also been attributed to the action of bursicon namely postecdysial reduction in blood volume due to enhanced diuresis (*P. americana*, Mills and Whitehead, 1970) and tracheal air-filling (*L. migratoria*, Vincent, 1971). Since these processes are open to serious criticism (Seligman, 1980) and based on circumstantial evidence respectively, they will not be discussed further.

Bursicon's properties are consistent with it being a large peptide, e.g. it is destroyed by the proteolytic enzymes trypsin and pronase, and by heating (Fraenkel and Hsiao, 1965). A number of attempts have been made to purify the hormone without success, presumably because it is unstable

when purified (see review by Seligman, 1980), though values for its molecular weight have been proposed (Reynolds, 1983).

The mode of action of bursicon as the initiator of cuticular tanning is little understood. *In vitro* experiments by Mills and Whitehead (1970) and Post (1972) are consistent with the hypothesis that bursicon increases the permeability of the blood cells to tyrosine. However, Seligman *et al.* (1969) have additionally suggested that the hormone promotes the utilization as well as the synthesis of DOPA. The transmission of the hormonal stimulus to the target tissue may occur via cAMP. The last named can mimic the effects of bursicon in newly emerged flies (Seligman and Doy, 1972) and cockroaches (Vandenberg and Mills, 1974) while the phosphodiesterase inhibitor, theophylline, synergises the hormone's action (Seligman and Doy, 1972). The most convincing evidence for the involvement of cAMP has been provided by Delachambre *et al.* (1979a) who demonstrated that maximal cAMP titres of the pharate adult epidermis of *Tenebrio molitor* preceeded high titres of bursicon in the blood, while bursicon containing material elevated cAMP in the epidermis (Delachambre *et al.*, 1979b).

Adult *Manduca sexta* are not competent to respond to bursicon until about 9 h before it is normally released (Reynolds *et al.*, 1979), pupae also will not respond to

bursicon until 2-6 h prior to release (Reynolds, 1983). The proximate cause of the acquisition of bursicon responsiveness is not known, although it may depend on the fall in the titre of haemolymph ecdysteroids which occurs before ecdysis, and which appears to be essential for the release of eclosion hormone (Truman, 1981) and the coordination of other developmental events which precede ecdysis (J.W. Truman, personal communication).

The above review of the literature has shown that rapid post-ecdysial tanning of the cuticle is controlled by the peptide hormone bursicon in most insects which have been studied. The hormone is released massively into the blood during or immediately after ecdysis and is thereafter rapidly eliminated. It is conspicuously absent from the blood at other times.

There are two apparent exceptions to this general pattern. In the first case Post and de Jong (1973) suggested that bursicon is present in the blood of pharate 5th instar larvae of *Pieris brassicae* well before the start of ecdysis. However, they used changes in concentration of free tyrosine in the blood as an indirect measure of bursicon activity. Their experiments are hardly conclusive and Reynolds (1983) has suggested that their results are open to alternative explanation. The second case concerns the yellow flour beetle *Tenebrio molitor*. In a comprehensive series of

papers, Delachambre and his colleagues (Delachambre, 1971; Grillot *et al.*, 1976; Delachambre *et al.*, 1979a,b) have shown that bursicon may indeed be released pre-ecdysially. Moreover, they suggested that the hormone is released over a period of about 2 days rather than in a single brief pulse.

Their model is based on two lines of evidence. In ligature experiments, Delachambre (1971) showed that normal tanning of the thoracic and abdominal sternites was prevented by either neck or thoracic/abdominal ligatures placed prior to the 4th day of pupal life. However, after day 6 tanning could not be prevented. This critical period implied the release of a tanning hormone well before ecdysis (which occurred on day 9). Evidence supporting this idea was obtained from measurements of bursicon in the blood and in the central nervous system. A conspecific bioassay was used in which isolated abdomens received injections of blood or tissue extracts at about the time of adult ecdysis. The tanning factor was present principally in the thoracic and abdominal ganglia of the ventral nerve cord and their associated neurohaemal organs (perivisceral organs), from which it was apparently released at about the time of the critical period (Grillot *et al.*, 1976). The tanning factor was detectable in the blood from this time, rising gradually to a peak titre at the time of ecdysis (Delachambre *et al.*, 1979a).

This scheme for the control of tanning in *Tenebrio* appeared so completely at variance with what is known for other insects that it was resolved to reinvestigate the problem using the classical adult blowfly bioassay devised by Fraenkel and Hsiao (1965).

Materials and Methods

The animals employed and the treatment of experimental animals were as described in the general materials and methods.

Scoring systems for tanning, elytra expansion and shedding of exuvia

Normal and ligated insects were scored for the above characters either 2 or 4 days after ecdysis (indicated in the text) using the scales shown in Tables 1 and 2.

Bioassay for bursicon

Larvae of the blowfly, *Calliphora vomitoria* Meigen, were obtained from a local fishing bait supplier, and allowed to pupate in sawdust. In order to synchronise adult eclosion, the pharate adults were kept from 1-7 days in the refrigerator following the emergence of the first few flies in each "batch". On return to room temperature most of the remaining flies

eclosed within the first few minutes.

Blood and tissue (CNS) samples were taken using the techniques described in the general materials and methods and stored at -20°C until required. Activity was not affected by freezing (see Table 3). Bursicon was detected by bioassay on neck-ligated adult blowflies, using essentially the procedure described by Fraenkel and Hsiao (1965). Blowflies were neck-ligated without anaesthetic as they emerged from their puparia, ensuring that the ptilinum was filled with blood. The ligature prevents the release of bursicon and thus post-ecdysial tanning. The test insects were left for 2 - 3 h at room temperature prior to injection so that the small proportion of flies which tanned (due to poor ligatures) could be discarded. Injections (2 µl) of blood or tissue extracts were made into the scutellum of ether-anaesthetised flies using a calibrated glass needle attached to a mouthpiece by a rubber tube. Flies treated in this way did not bleed after injection, and so no attempt was made to seal the wound at the injection site. The injected flies were kept for 3 h at room temperature and then immersed in 70% ethanol to terminate the assay and render cuticle coloration more easily visible. Tanning of the thoracic and abdominal sclerites was assessed soon after the termination of the assay using a four-point scoring system similar to that used by Vincent (1972; see Table 4). In order to quantify bursicon, blood and tissue samples were appropriately

diluted so that the tanning response obtained in the assay was on the linear portion of the dose-response curve (see Figs. 1 and 2). In practice, a score of 2.5 was the highest accepted. Bursicon activity was then computed as a "relative score", being the actual score achieved in the bioassay multiplied by the dilution of the sample.

Determination of the rate of abdominal pumping

Pharate adult *Tenebrio molitor* of appropriate age and treatment were placed in a copper water jacket at 25 ± 0.5°C, a 1.5 cm thick perspex lid was replaced and the insects were left to acclimatise for approx. 30 minutes. Individual insects were then observed through the transparent lid of the water jacket with the aid of a binocular dissecting microscope (Zeiss) and the abdominal flexions (= pumping) tallied over a period of time (normally 10 minutes) using an event recorder.

Measuring extensibility of the elytra

The technique employed was essentially that described by Reynolds (1977). An elytron was removed from a pharate adult *Tenebrio* of appropriate age and treatment. The exuvial cuticle was peeled carefully away and two marks of "humbrol" paint were placed, with the aid of an eye lash, exactly 5 mm apart on the leading edge of the new adult cuticle.

A 0.3 g paper clip was attached to the distal end of the elytron with "super glue". Provisional experiments showed this weight to be optimal in providing a reasonable degree of extension over a 15 min period. The preparation was now suspended from a "bulldog" clip and viewed through a binocular microscope (Zeiss). The increase in the distance between the two paint marks with time was determined with the aid of a calibrated eye piece graticule.

Table 1. Tanning score. - refers to the ventral sternites

A. 5-point system*

0	- untanned
1	- mostly tanned with light brown patches
2	- mostly light brown with untanned patches
3	- uniformly light brown
4	- uniformly dark brown

* similar to that employed by Delachambre (1971) with the difference that the patchy tanning has been divided into two classes (1 and 2) and not kept as one.

B. 7-point system

0	- untanned
1	- small patches
2	- < 50% tanned
3	- >50% tanned
4	- 90% tanned (only few small patches untanned)
5	- uniformly light brown
6	- uniformly dark brown

Fig. 1. Bursicon dose response curve.

Dilutions prepared from pooled samples of blood taken from 1 h -old adults

Legend: Abscissa: µl of blood, made up to 2µl with E/B saline

Ordinate: tanning score

: Vertical lines denote SE of the mean

Figures indicate the number of determinations.

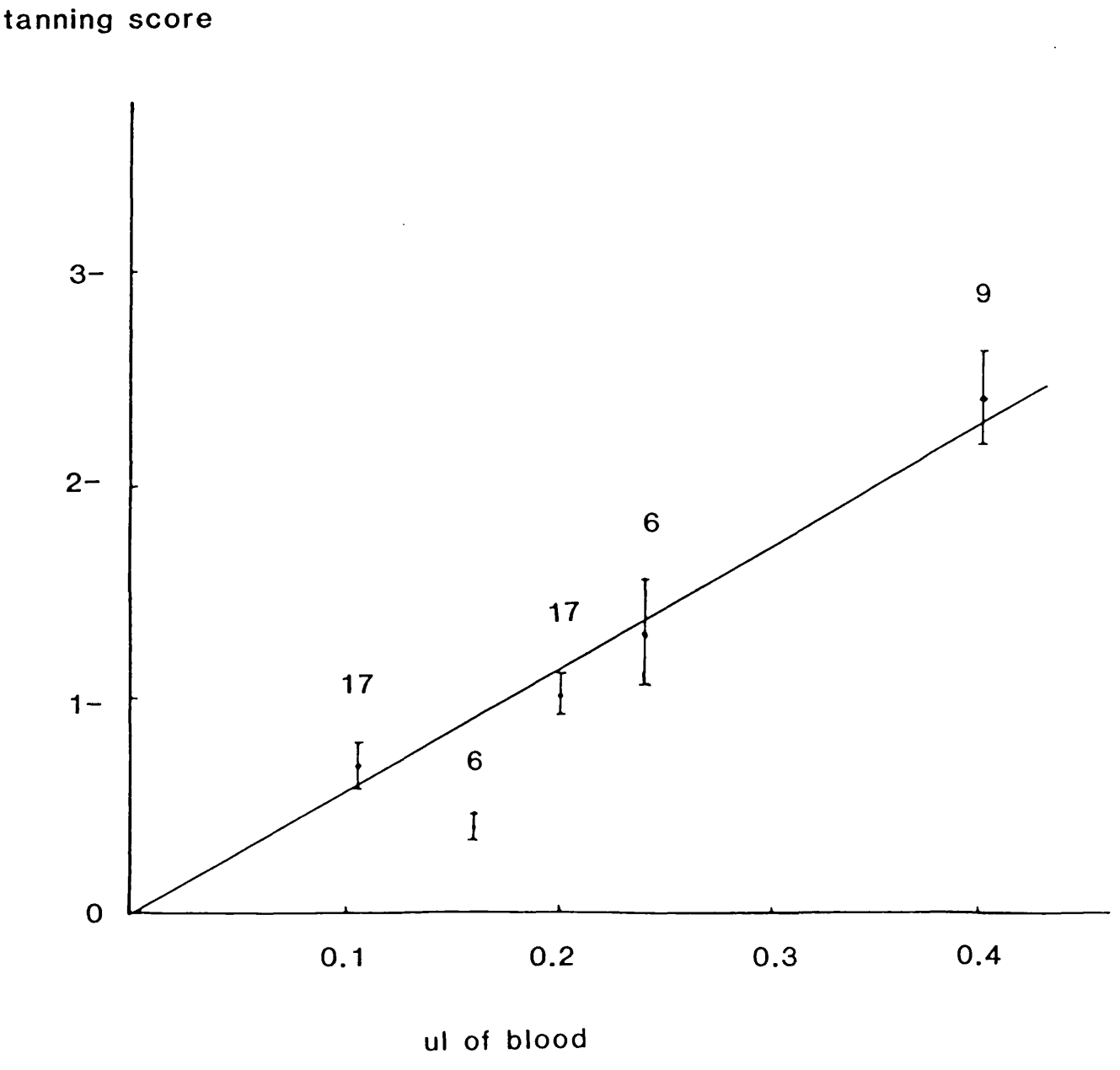

tanning score
3-
2-
1-
0
17
6
17
6
9
0.1
0.2
0.3
0.4
ul of blood

Fig. 2. Bursicon dose response curve.

Dilutions prepared from pooled samples of blood taken from 2 h-old adults

Legend: Abscissa: µl of blood, made up to 2 µl with E/B saline

Ordinate: tanning score

Vertical lines denote SE of the mean

Figures indicate the number of determinations

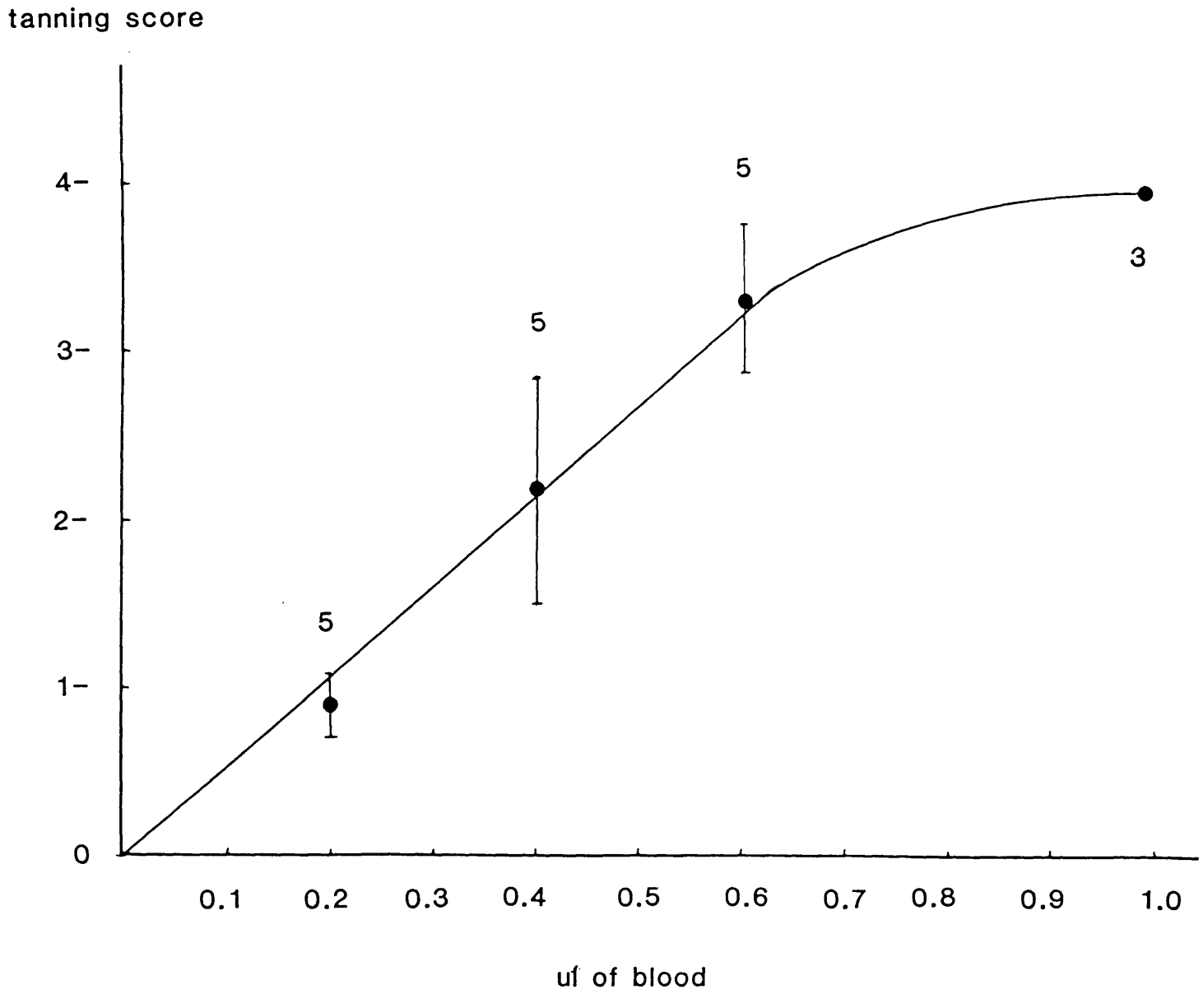

tanning score
4-
3-
2-
1-
0
5
5
5
3
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
ul of blood

Table 2.

A Elytra expansion score

0	elytra completely unexpanded
1	elytra ½ expanded
2	elytra completely expanded

B Shedding of exuvia score*

0	exuvia completely unshed
1	exuvia ½ shed
2	exuvia completely shed

* also referred to as ecdysial score.

Table 3. Effect of freezing on bursicon activity in the blood of *Tenebrio molitor*

Blood was extracted from newly ecdysed adults 30 min after the start of ecdysial behaviour. The pooled extract was divided into two. One aliquot was appropriately diluted with Ephrussi-Beadle Saline (E/B) and assayed for bursicon. The other was placed at -20°C for 2 h prior to thawing, dilution and assay.

Treatment	Dilution (µl of blood diluted with E/B to give 2 µl)	Tanning score		N
		Mean	SE	
Frozen blood	0.1	1.94	0.35	9
	0.2	2.9	0.25	10
Fresh blood	0.1	1.95	0.29	11
	0.2	3.3	0.1	11

Statistical analysis

	t	P	
frozen v fresh (0.1 µl)	0.022	0.9	not significant
frozen v fresh (0.2 µl)	1.487	0.2-0.1	not significant

Table 4. Bioassay for bursicon: blowfly tanning score.

- refers to dorsal surface

0	-	no tanning
0.5	-	small patches on either thorax or abdomen
1.0	-	small patches on both thorax and abdomen
1.5	-	< 50% patch on either thorax or abdomen
2.0	-	< 50% patch on both thorax & abdomen
2.5	-	> 50% patches on either thorax or abdomen
3.0	-	> 50% patches on both thorax and abdomen or thorax or abdomen completely tanned
3.5	-	thorax or abdomen completely tanned and >50% patch on the other
4.0	-	both thorax and abdomen completely tanned.

Results

The effect of neck-ligation on tanning

Cotton ligatures were applied to the necks of pupae at different times prior to the adult moult. Insects were scored for tanning 48 h after ecdysis using the 5-point scoring system (see Materials and Methods). The results are shown in Fig. 3. Individuals ligated at 1 - 3 h after the pupal moult exhibited the lowest tanning score. Those insects operated on between days 2 - 8 of pupal life showed a variable measure of tanning, but significantly more than 1 - 3 h ligated animals (apart from on day 8, see legend to Fig.3). However, insects ligated 1 h before the adult ecdysis on day 9 tanned normally, achieving a score which was little different from normal and significantly larger than all other operated groups ($P < 0.001$).

Neck ligation of 1 - 3 h old pupae had no effect on the timing of the adult ecdysis. However, if the operation was performed on older pupae the adult ecdysis was delayed: pupae ligated when 24 h old ecdysed 1 - 2 days late to adult; pupae ligated on days 2 - 6 ecdysed 3 - 5 days late; pupae ligated on day 7 ecdysed 1 - 2 days late; pupae ligated on day 8 ecdysed approx. 6 h late. These observations on the effects of neck-ligation on the timing of adult ecdysis are based on hundreds of operations. In contrast to the above Delachambre (1971) reported that neck ligation affected the time of ecdysis of his experimental insects as

follows: ligation of pharate pupae at the moment of ecdysis delayed adult ecdysis by 20 - 25 days; pupae ligated at 15 min - 12h ecdysed 5 - 10 days late; pupae ligated up to day 6 ecdysed 2 - 5 days late.

In the present work, young pupae (1 - 3 h old) and "old" pupae (8 days old) withstood the trauma of the operation. However, many of the 1 - 7 day old pupae failed to survive the operation (mortality was often in excess of 90%) due to tearing of the cuticle and subsequent exsanguination.

Young pupae (1 - 3 h old) were used routinely for neck ligation experiments. It is interesting to note that these insects produced a new adult cuticle on the proximal side of the ligature and the pupal head was shed at the adult ecdysis. In some cases these insects produced part of an adult head. This took the form of either a formless but articulated (with a "neck") stump or part of a mandible. These insects were not dissected, therefore it is not known whether their nervous systems had managed to regenerate. However, their behaviour and appearance was no different to that of the majority of the ligated insects which did not produce a reduced adult head.

It would seem that normal tanning in the adult is dependent on the presence of the head during the 24 h prior to the ecdysis. In order to time the critical period for

the head more precisely neck ligatures were placed at different times during the last 24 h of pupal life. In contrast to the 1st experiment, when tanning was assessed after 48 h (Fig. 3) in this experiment , insects were scored for tanning 4 days after ecdysis. However a direct comparison can be made between the two experiments, because though tanning score does increase with time after ecdysis (see Fig. 4A), the change between 2 and 4 days is not significant (P = 0.4 - 0.3).

The results of the second experiment are shown in Fig. 5, from which it is clear that the critical period for the head in tanning is between 8 and 24 h prior to the ecdysis, with an approximate RT_{50}* of 14 h. This is very different from the experiments of Delachambre (1971) in which neck or thorax ligated insects failed to prevent normal tanning when placed after day 6 of pupal life. There is no obvious reason for this discrepancy.

*RT_{50} = time of the operation resulting in a tanning score which was 50% of the maximum.

The staging system outlined in Table 1 of the general materials and methods was clearly inadequate for experiment 2 (Fig. 5), because ligatures were to be placed at frequent intervals during the 24 h prior to adult ecdysis. Therefore the stage at which neck ligation was performed was determined retrospectively by noting the time of onset of ecdysial behaviour. Since neck ligation delays ecdysis, the critical period and the RT_{50} for the head in tanning are inaccurate. The delay is approximately 6 h when neck ligation is performed at -24 h and is progressively reduced as the operation is carried out closer to ecdysis. Therefore the actual RT_{50} for the operation is ≃ - 11 h rather than - 14 h, and the critical period is ≃ -18 h to - 6 h rather than - 24 h to - 8 h.

The scoring system employed in these experiments was similar to that used by Delachambre (1971 ; see Table 1). However, Delachambre *et al.* (1979) revised their system, such that the tanning score of a cohort was expressed as the % of class 3 and class 4 individuals among the total. When the results in Fig. 3 are re-expressed using this

system, we find that the pattern is broadly the same, though the tanning score of young ligated pupae (days 2 and 4) is increased with respect to that of animals operated on day 9 (Fig. 6).

Ecdysial behaviour in pharate adult *T. molitor*

The brief description of ecdysial behaviour given in the legend to Fig. 8 is based on observations made on some 40 individuals. The only other published details on ecdysial behaviour in *Tenebrio* are those given by Provansal *et al.* (1977). The two descriptions are broadly comparable apart from the failure of Provansal *et al.* (1977) to note that the "abdominal rotation response" (aar)* (see Fig. 8; stage 1: rolling) heralds the start of ecdysial behaviour. aar occurs throughout pupal life and Askew and Kurtz (1974) suggest that it functions as "1) a means of defense from external threats (parasites, predators etc.); 2) a locomotor device to escape unfavourable environmental conditions; and 3) as an aid to physical emergence from the pupal skin." They found a large increase in the frequency of spontaneous aar's during the last 24 h of pupal life, but made no close observations around the time of ecdysis. In the present work, an aar* invariably preceded the straightening of the abdomen which confirmed the start of ecdysis.

* a vigorous circular movement of the abdominal segments.

Haemolymph and nerve cord titres of bursicon in *Tenebrio molitor*

In preliminary experiments, Fraenkel and Hsiao (1965) established that the blood of ecdysing adult mealworms contained a factor which was active in the blowfly bioassay for bursicon. This rapid and simple method was chosen to quantify the hormone in both blood and tissues of *Tenebrio*, because considerable doubts were entertained about the validity of the conspecific bioassay of Delachambre as a measure of bursicon (see Discussion).

First it was established that bursicon is present in the blood only at the time of ecdysis (see Table 5). Whereas considerable amounts of the hormone could be detected in the blood of ecdysing pupae and adults using the blowfly bioassay, the extent of tanning in flies which received blood from inter-moult pupae and pharate adults was not significantly different from that produced by injections of saline alone.

Release of the hormone was a brief event. Maximum titres were achieved in insects sampled 30 min after the start of ecdysis. The hormone subsequently disappeared rapidly from the blood, with an approximate half life of 1 - 2h(see Fig. 7). The extreme rapidity of bursicon release was established by taking blood samples from individuals at closely observed stages of ecdysis (see Fig. 8). A small amount of hormonal activity was detectable in the blood of insects which were bled at the first sign of ecdysial behaviour. Since it took a few seconds to bleed the insects, this probably means that the release of

bursicon begins at the same time as overt ecdysis behaviour. Bursicon activity was never found in pharate adults which had completed resorption of moulting fluid but which had not yet begun ecdysis. By the time the old cuticle was split, only 2 min after the beginning of ecdysis, the titre of bursicon in the blood had already reached 60% of the maximum value that was recorded.

The pattern of release at pupal ecdysis was similar in every respect, although the maximum titre achieved was somewhat lower than at adult ecdysis (Fig. 9).

All parts of the CNS examined were found to contain some bursicon activity detectable by the blowfly bioassay. However, more than 90% of the total activity was present in the thoracic and abdominal ganglia of the ventral nerve cord (TG and AG respectively). Titres of hormone in these tissues declined markedly during both pupal and adult ecdysis, presumably denoting its release into the blood (Fig. 10). By contrast, the titres measured in the CNS during pupal and pharate adult development remained constant.

The amounts of bursicon which disappear from the CNS during pupal and adult ecdysis are insufficient to account for all the hormone which appears in the blood. It is possible that this is due to the fact that the PVOs were deliberately excluded from the CNS samples, because it is difficult to

ensure that they are dissected away from surrounding tissues in a uniform way. However, when attempts were made to assay thoracic and abdominal ganglia together with their PVOs (preliminary observations) there was no great increase in the amount of bursicon activity. Thus it is possible that the hormone is modified in some way as it secreted, so as to increase its bioactivity.

The above findings do not support Delachambre's suggestion that in *Tenebrio* "bursicon" is released considerably before adult ecdysis. The discrepancy between these findings and his may be explained by making the hypothesis that Delachambre's critical period on days 4 - 6 ($-^{18}$ h → $-^{6h}$ in the present work, see Fig. 5) represents not the release of bursicon itself, but another, previous endocrine event which is required for bursicon's release. This hypothesis was tested by measuring bursicon titres during ecdysis in insects which had been neck-ligated either 1 - 3 h after pupal ecdysis or 24 h before the adult ecdysis. As expected, both groups of ligated insects showed very little of the cuticle tanning which normally occurs after adult ecdysis, even though their pre-ecdysial tanning was similar to that seen in unligated insects. It is clear from Fig. 11 and Table 6 however, that bursicon release occurs normally in both experimental groups. Indeed paradoxically the bursicon titre in -24 h ligated insects, 45 mins after the onset of ecdysial behaviour, was significantly larger than that in normal insects at a

comparable time. The time course of bursicon activity in the blood of ecdysing 1 - 3 h ligated adults (as measured by the blowfly bioassay) (Fig. 11) was almost identical to that measured in intact insects (Fig. 7).

Since bursicon is released normally in neck-ligated insects which do not tan, then Delachambre's factor (referred to here as DF) cannot act to promote or permit the release of bursicon. On the other hand, DF could act to promote competence of the epidermis to respond to bursicon when this is subsequently released at the time of ecdysis. Assuming that the critical period for the head in tanning shown in this study corresponds to that reported by Delachambre (1971) (though the timing of the two are very different, see earlier), it follows that in Bath insects DF is released during the 18h prior to ecdysis.

The hypothesis was made that a factor is released into the blood of pharate adult *Tenebrio* during the 18 h prior to ecdysis which confers competence to respond to bursicon. This hypothesis was tested in the following experiments.

Effect of blood transfusions and nerve cord extracts on the tanning score of neck-ligated and thorax-ligated *T. molitor*

Insects neck-ligated 1 - 3 h after the pupal ecdysis then injected 12 h or 3 h prior to adult ecdysis with blood taken from normal insects at these times had significantly greater tanning

scores than the non-injected controls (Table 7; column 3; and Table 7a). A similar result was achieved using insects that had been neck-ligated 24 h prior to the adult ecdysis, then injected 1 - 3 h before ecdysis with blood taken from eclosing adults (Table 8). However, in all cases saline injected controls tanned to the same extent as those injected with blood, suggesting that enhanced tanning in both experimental groups was the result of injury caused by the injection (see Tables 7, 7a and 8).Support for this conclusion comes from experiments in which insects were injured by pricking with a needle at an appropriate time prior to ecdysis (see Tables 9 and 10). The mean tanning score of such experimental groups was not significantly different from saline-injected controls ($P > 0.1$).

In conclusion, injecting aliquots of blood from normal pharate adults into neck-ligated pharate adults did not promote competence to respond to bursicon. By contrast Delachambre (1971) found that blood taken from pupae within 2 days of the adult moult induced normal tanning in neck-ligated insects, thus demonstrating the presence of DF in the blood of "late" pupae.

Failure to demonstrate the presence of a tanning promoting factor in the blood of pharate pupae during or just after the critical period for the head in the present experiments could be due to one or more of:-

1. The bioassay (neck-ligated insect) although similar to that employed by Delachambre (1971) was not sensitive enough to respond to the level of hormone present in a small volume of blood (4 µl) taken from a "Bath University" *Tenebrio*.
2. Blood samples were taken at the wrong time i.e. when the hormone was absent from the blood. Timing of sampling would be particularly important if the factor has a short ½-life in the blood and its release is a brief event. If the dynamics of the release of DF in "Bath University" *Tenebrio* conformed to this pattern, it would make them very different from those used by Delachambre and his colleagues in which DF was present in the blood for a period of days.
3. Test ligated insects were used at times when they were not responsive to DF i.e. responsiveness is restricted to a brief period. Once again, if true, this would make the insects used in the present work very different from those employed by Delachambre, which were responsive to DF over a period of 2 days.

An attempt was made to increase the discrimination of the bioassay by modifying the tanning-score system as follows:

i. expressing the results as % of insects with class 3 and 4 scores among the total;

ii. scoring insects on an expanded 7-point scheme (see Table 1B) rather than the routinely used 5-point

scheme (see Table 1A).

The results of the experiment shown in Table 7, column 3 have been re-expressed as in (i) and (ii) above. However, neither modification affects the outcome (see Table 7 ; columns 4 and 5; and Table 7a). In all three systems (compare columns 3, 4 and 5 of Table 7), injections of blood and saline caused an increase in tanning score over that achieved by non-injected controls, but there was little difference between saline and blood injected treatments.

An additional approach to improving the bioassay is the use of isolated abdomens (Delachambre *et al.*, 1979). However injection of - 3 h pharate adult blood into the abdomens of thorax-ligated insects of similar age did not increase the tanning score above that of the injured controls (Table 9). Inexplicably saline-injected controls had a significantly greater tanning score than insects injected with blood ($P = 0.01 - 0.001$).

The failure of blood transfusions to induce competence to respond to bursicon could be a reflection of a low titre of DF in the blood of the insects used (see above). The nerve cord is an alternative source of DF, indeed Delachambre (1971) and Grillot *et al.* (1976) found large amounts of DF activity in the nerve cord of *Tenebrio* pupae. Unfortunately, once again the age of the tissue at the time of extraction appears

to be critical. Grillot *et al.* (1976) found a large drop in nerve cord DF activity at the time of release into the blood (within the last 2 days of pupal life). In the present study, the time period of release of DF, as judged by the critical period for the head, was considerably later than that observed by Delachambre (1971) and Grillot *et al.* (1976). Consequently the assumption was made that no fall in nerve cord DF titre would occur until ca. -18 h and, with this in mind, an extract was made from the nerve cords of -24 h insects. The extract was injected into 1 - 3 h neck-ligated pharate adults 3 h prior to ecdysis. The tanning score of the recipients was not significantly different from that of injured controls (Table 10). The same was true of the effects of an extract from -6 to -12 h nerve cords injected into 1-3 h neck-ligated insects 18h prior to the ecdysis. Insects which had been injected with -24 h nerve cord extract exhibited a tanning score that was not significantly different from non-injected controls ($P = 0.1 - 0.05$), or from saline-injected insects ($P = 0.1 - 0.05$).

Failure to demonstrate the presence of DF in blood and nerve cord extracts, in spite of the modifications to the technique described, is at variance with Delachambre (1971) and Grillot *et al.* (1976) who used comparable techniques to those employed here. The reason for the discrepancy between the present work and that of Delachambre and his colleagues is not immediately apparent . However, since there are no

positive reasons for doubting the findings of Delachambre's group, the existence of DF is assumed in the following experiments, as is its place of origin, the nerve cord.

The identity of DF, if as is argued here it is not bursicon, remains to be established. An attempt has been made to find other events with the same critical period for the head as that for tanning. It was hypothesised that if DF co-ordinates other activities around the time of ecdysis, then the discovery of the nature of these phenomena might give a clue to the identity of DF.

The effect of neck ligation on the rate of abdominal pumping

Pupal stages of a number of insect species show rhythmic pulsations of haemolymph pressure (Slama, 1976). Provansal *et al.* (1977) has suggested that the pulses may facilitate tracheal ventilation, aid circulation of the haemolymph and play a central role in ecdysis. They divided the pattern of pulses into 5 categories, the last of which ("type F") predominates prior to adult ecdysis and is associated with pumping movements of the whole abdomen. Continuous recording of the pressure pulses during the 2½ h prior to the onset of ecdysial behaviour revealed a gradual increase in mean haemolymph pressure and amplitudes of abdominal flexions. Subsequently, peristaltic waves of contraction of abdominal muscles brought about a 2 - 3 fold increase in haemolymph

pressure and movement of haemolymph from abdomen to thorax. These events are responsible for the rupture of the exuvia along the dorsal suture (Provansal *et al.*, 1977). Provansal *et al.* (1977) suggested that the neuromuscular events responsible for the changes in haemolymph pressure are orchestrated by neurohormone(s). If DF is involved then neck ligation should interfere with the events described above, with a critical period for the head similar to that in tanning. Unfortunately equipment for measuring haemolymph pressure was not available (e.g. see Slama (1976)), consequently abdominal pumping was taken as a measure of the frequency of "type F" haemolymph pressure pulses.

The frequency of abdominal flexions was recorded at strategic points during the 24 h prior to ecdysis (Fig. 12). These measurements show for the first time a large significant increase in the rate of abdominal pumping in the 4 h before ecdysis. Pupae which had been neck-ligated either 1 - 3 h after the pupal ecdysis or 24 h before the adult ecdysis failed to show this increase in the rate of abdominal pumping. The number of flexions/minute performed by 1 - 3 h ligated insects did not increase significantly between - 12 h and - 3 h, and declined markedly by -1 h. It is interesting to note that the rate of abdominal pumping in insects ligated at 18 h before adult ecdysis was significantly greater than that of -24 h ligated individuals recorded 1 h before ecdysis.

The effect of neck-ligation on the ability of adult *T. molitor* to shed the exuvia

It seemed likely that the failure of neck-ligated insects to increase the rate of abdominal pumping, and thus presumably their haemolymph pressure, might interfere with their ability to shed the exuvia. To investigate this possibility neck ligatures were placed at different times during the last 24 h of pupal life. Operated insects were subsequently scored for the extent of removal of the exuvia (see Materials and Methods for the scoring system). Normal shedding of the exuvia proved to be dependent on the presence of the head during the 24 h prior to ecdysis (Fig. 13). The shape of the graph is different from that produced by the effect of neck ligation on tanning (see Fig. 5). The critical period for the head in shedding of the exuvia,* - 24 h → -1 h with ca. RT_{50} of - 4 h, is somewhat different from that in tanning, -18 h → - 6 h with ca. RT_{50} of -11 h.

Effect of neck-ligation on elytra cuticle extensibility

Expansion of the new adult cuticle during and after ecdysis in a number of species of insect has been shown to be facilitated by a temporary increase in the cuticle's extensibility. This change in mechanical properties is termed plasticization (Reynolds, 1983). In the tobacco hornworm, *Manduca sexta* the wings exhibit two successive periods of increased extensibility (Reynolds, 1977). The first occurs

* taking into account the delaying effect of neck-ligation on ecdysis (see page 27).

prior to adult ecdysis and is induced by the eclosion hormone, and the second , controlled by bursicon, occurs post-ecdysially at the time of wing spreading.

Reynolds (1977) determined extensibility by measuring the increase in distance between two paint marks on a wing under an "imposed load". This technique has been employed here to monitor any change in extensibility of the elytra of pharate adult *Tenebrio*. The responses of elytra from normal and neck-ligated pharate insects have been compared.

The increase in length of the wing following the application of the 0.3 g weight was time dependent (see Figs. 14, 15, 16). A similar situation was found for wings of *Manduca* (Reynolds, 1977). Elytra from normal animals showed a large increase in extensibility between -18 h and -12 h from the adult ecdysis (Fig.14).

In order to try and define more precisely the time of onset of plasticization of the elytral cuticle, extensibility of the elytra was determined at more closely observed stages of development (Fig. 18). It would seem that plasticization starts around 18 h prior to ecdysis (see Fig. 18 and Table 1 in the Materials and Methods).

This plasticization of the elytra cuticle was dependent on the presence of the head. No increase in extensibility

occurred if pupae were neck-ligated at either 1 - 3 h after the pupal ecdysis (Fig. 15) or 24 h before the adult ecdysis (Fig. 16). For comparative purposes the extension of elytra after 15 minutes under load have been expressed as a % of the 0 time values and plotted as a histogram in Fig. 17. Statistical analyses of the data (see Fig. 17) confirm that a large significant increase in extensibility occurs between -18 and -12 h in normal animals, but not in ligated insects.

The effect of neck-ligation on post-ecdysial expansion of the elytra

Post-ecdysial expansion of the new cuticle as exemplified by the elytra must be affected by a number of factors, in particular haemolymph pressure and cuticular extensibility.

Applications of neck ligatures at different times within the last 24 h of pupal life revealed a critical period for the head in elytra expansion* viz. -24 to -1 h (Fig. 19). The shape of the graph is similar to that produced by the effect of neck ligation on ecdysial score (Fig. 13). The RT_{50}s of the two treatments are almost identical viz. - 3½h (elytra expansion) and - 4 h (shedding exuvia).

* taking into account the delaying effect of neck-ligation on ecdysis (see page 27).

Fig. 3. The effect of neck-ligation on tanning.

Legend: abscissa: time of the operation in hours or days after the pupal ecdysis

ordinate: tanning score 2 days after ecdysis (5-point scale, see Table 1A)

Vertical lines denote SE of the mean. Figures in parentheses indicate number of determinations.

Statistical comparisons of data		t	P	
1 - 3 h	v 2 days	6.941	< 0.001	highly significant
"	v 3 days	4.201	< 0.001	" "
"	v 4 days	6.757	< 0.001	" "
"	v 5 days	5.245	< 0.001	" "
"	v 6 days	3.363	0.01 - 0.001	significant
"	v 7 days	2.809	0.01 - 0.001	"
"	v 8 days	1.803	0.1 - 0.05	not significant
"	v 9 days	12.21	< 0.001	highly significant
9 days	v 2 days	7.289	< 0.001	" "
"	v 3 days	6.234	< 0.001	" "
"	v 4 days	7.032	< 0.001	" "
"	v 5 days	8.850	< 0.001	" "
"	v 6 days	10.154	< 0.001	" "
"	v 7 days	9.237	< 0.001	" "
"	v 8 days	5.565	< 0.001	" "

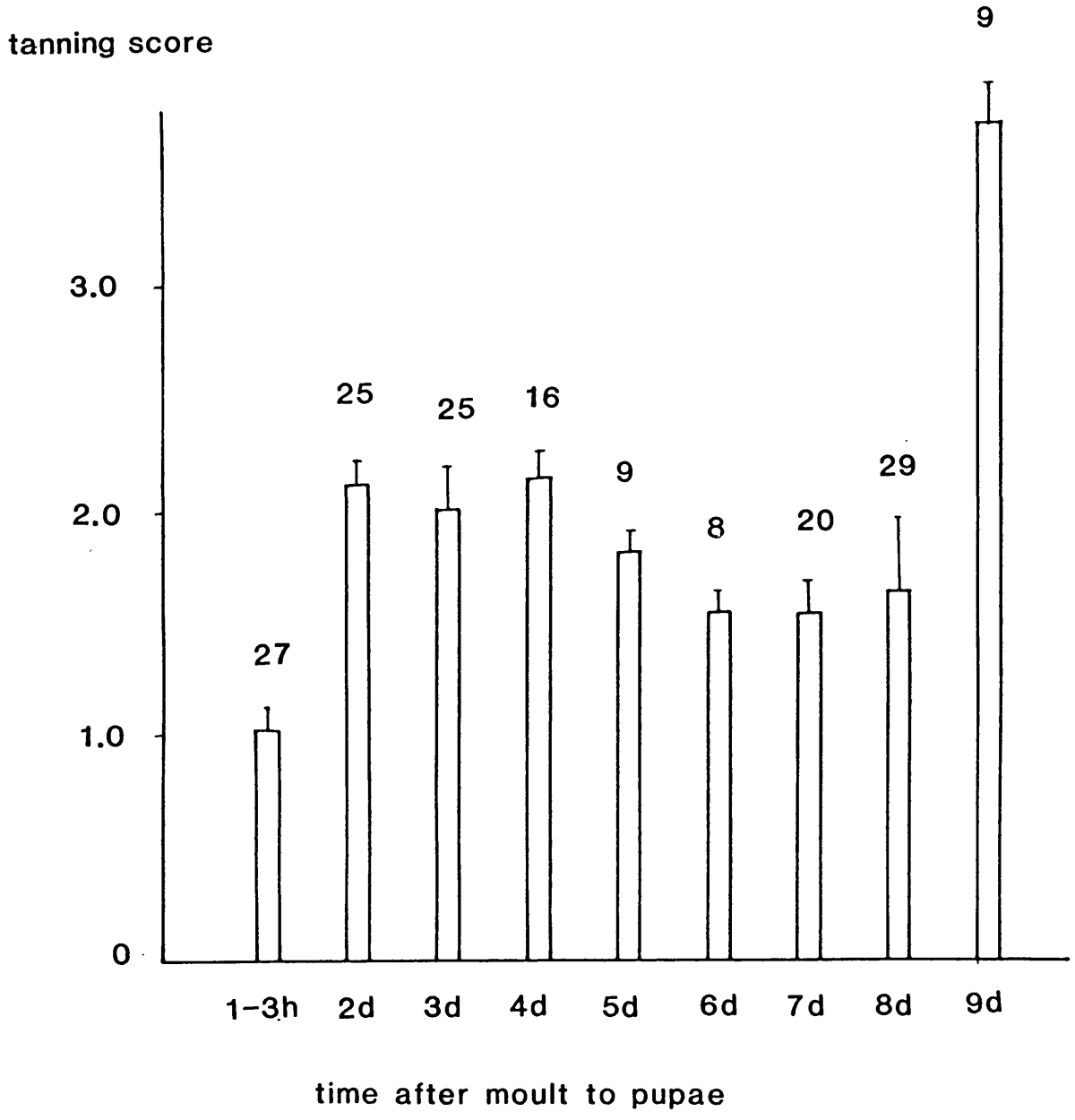

tanning score
3.0
2.0
1.0
0
27
25
25
16
9
8
20
29
9
1-3h
2d
3d
4d
5d
6d
7d
8d
9d
time after moult to pupae

Fig. 4. Effect of time and the scoring system employed on the tanning score

A 5 point scoring system (see Table 1A)

B 7-point scoring system (see Table 1B)

Legend: abscissa: time of scoring in days after ecdysis

ordinate: tanning score

Vertical lines denote SE of the mean. Figures indicate number of determinations

Statistical comparisons of the data	t	P	
A. 2 days v 4 days	0.900	0.4 - 0.3	not significant
B. 2 days v 4 days	0.708	0.5 - 0.4	not significant

-24 h lig. insect

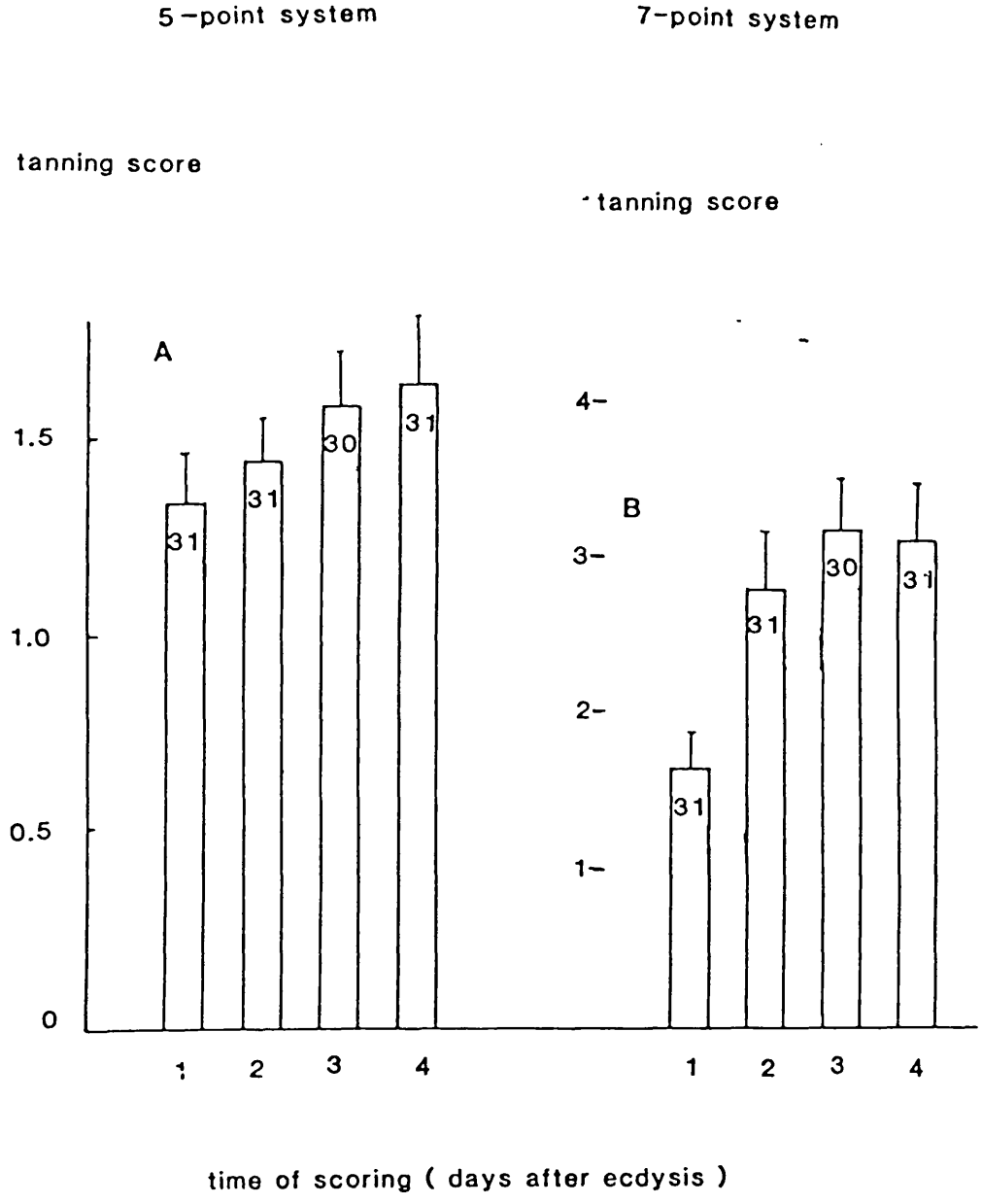


Fig. 5. Effect of neck-ligation on tanning.

Legend: abscissa: time of the operation in hours before the adult ecdysis

ordinate: tanning score 4 days after ecdysis (5-point scale see Table 1A)

Vertical lines denote SE of the mean.

Figures indicate number of determinations.

developmental stage at time of ligation determined retrospectively (see page 27).

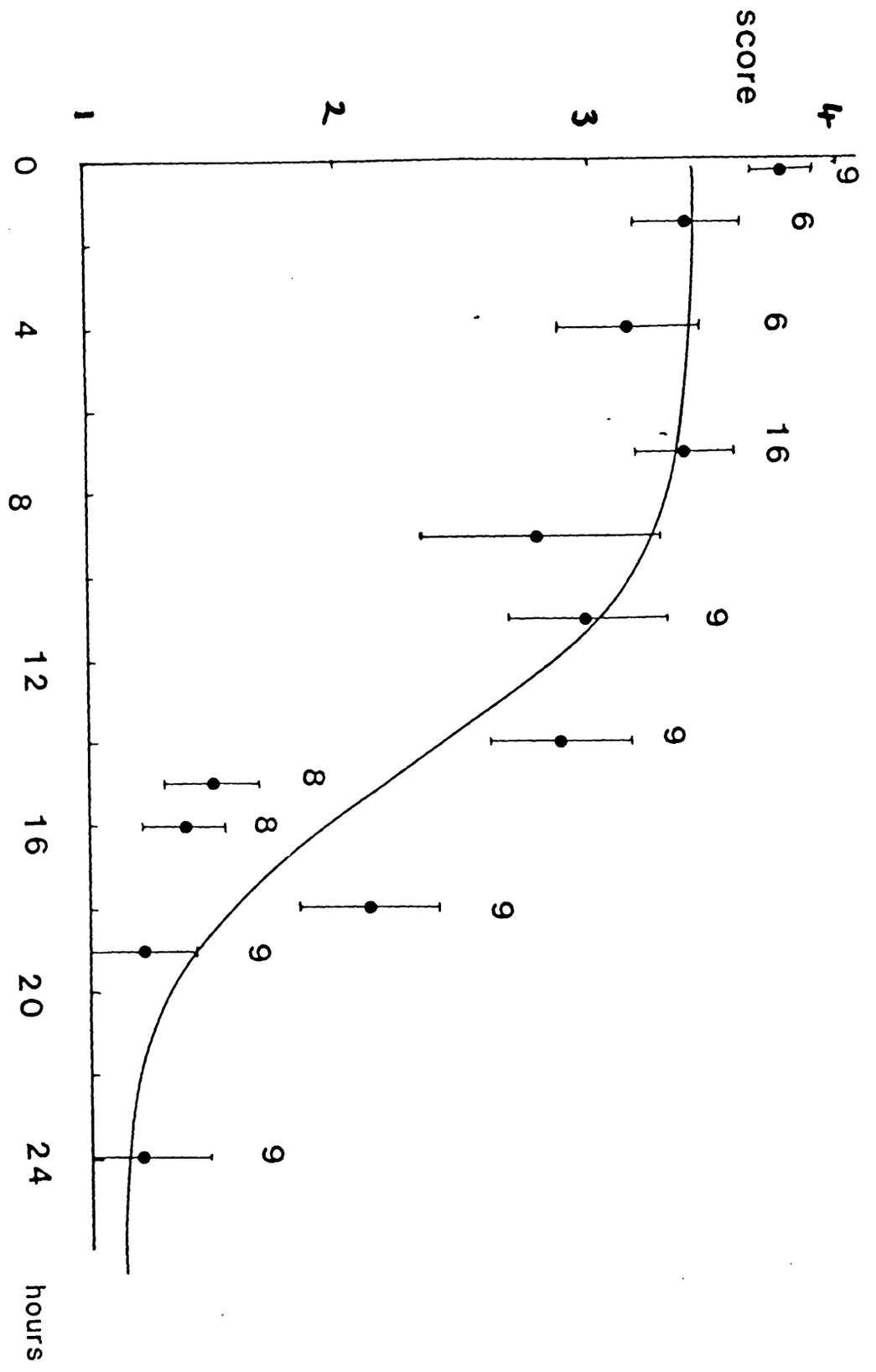

score
1
2
3
4
0
4
8
12
16
20
24
hours
9
6
6
16
9
9
8
8
9
9
9

Fig. 6. Results in Fig. 3, re-expressed as the % of class 3 and 4 individuals amongst the total number of experimental animals.

Legend: abscissa: time of the operation in hours or days after the pupal ecdysis

ordinate: % tanning score

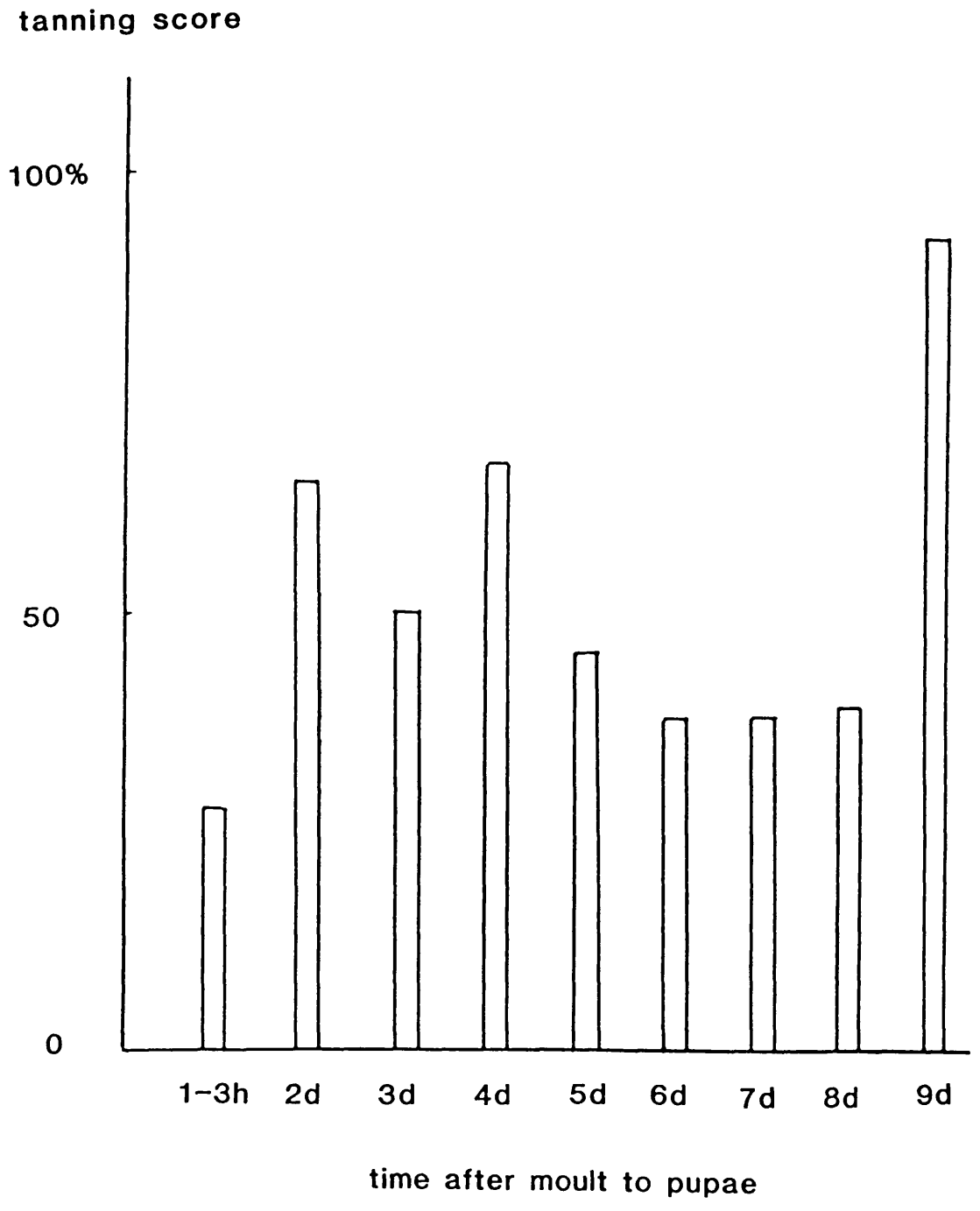

tanning score
100%
50
0
1-3h
2d
3d
4d
5d
6d
7d
8d
9d
time after moult to pupae

Table 5 . The haemolymph titre of bursicon in pupae and pharate adults of *Tenebrio molitor*.

Days after pupal ecdysis	Relative score		
	Mean	S.E.	N
0*	24.8	2.1	10
1	0.15	0.12	10
2	0.05	0.05	9
3	0.05	0.05	9
4	0	0	10
5	0.06	0.06	8
6	0.06	0.05	9
7	0.13	0.08	8
8	0.10	0.10	7
9*	33.0	0.5	11
control	0.07	0.07	7

* - sampled 30 min after the start of pupal ecdysis (day 0) or adult ecdysis (day 9). These gave mean values which were significantly different (t-test, $P < 0.001$) from the control (flies injected with 2 µl saline alone). Other samples gave mean values which were not significantly different from the control ($P > 0.6$)

Fig. 7. Bursicon activity in the blood of *Tenebrio molitor* during ecdysis

Legend. Abscissa: time in h after the start of ecdysis

Ordinate: relative score (= actual bioassay score multiplied by dilution of sample).

Results are means (± SE) of the number of determinations indicated. The continuous line is fitted by eye, and is drawn assuming that release of hormone begins at the start of ecdysis (see text).

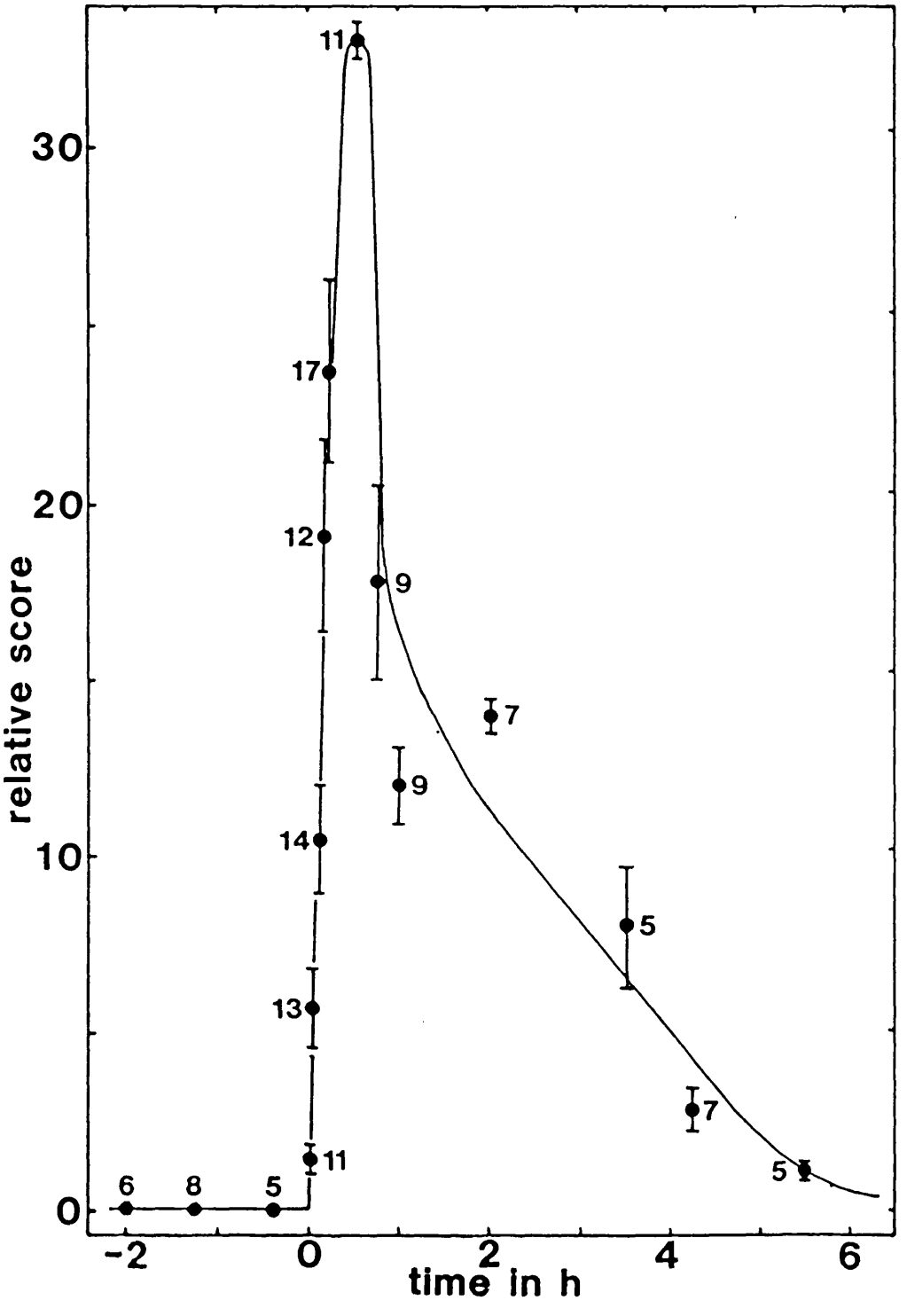

relative score
30
20
10
0
-2
0
2
4
6
time in h
11
17
12
9
7
9
14
5
13
7
11
5
6
8
5

Fig. 8. Correlation between blood titres of bursicon and behaviour during adult ecdysis in *Tenebrio molitor*.

Legend: Abscissa: stage 1 - rolling behaviour (aar)

stage 2 - abdomen straightens

stage 3 - backward movement of old cuticle (peristalsis)

stage 4 - splitting of old pronotal cuticle

stage 5 - exuvia shed

Stages 1-4 take about 2 min, stages 4-5 take about 12 min. Other details as for Fig. 7.

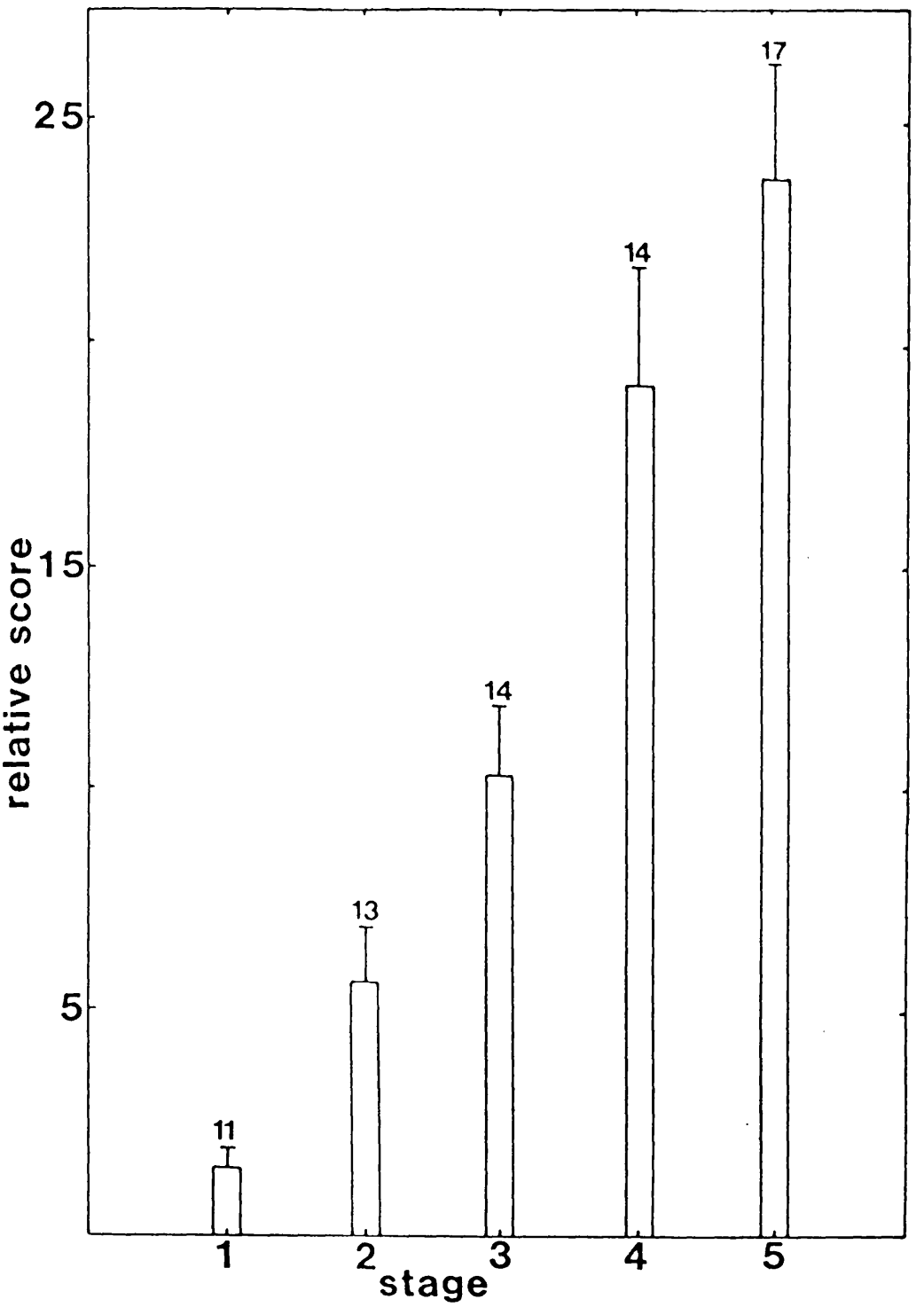

25
15
5
relative score
11
13
14
14
17
1
2
3
4
5
stage

Fig. 9. Bursicon activity in the blood of *Tenebrio molitor* during pupal ecdysis.

Legend:

P - pharate pupa (moulting fluid resorbed and therefore within a few hours of ecdysis).

Other details are as for Fig. 7.

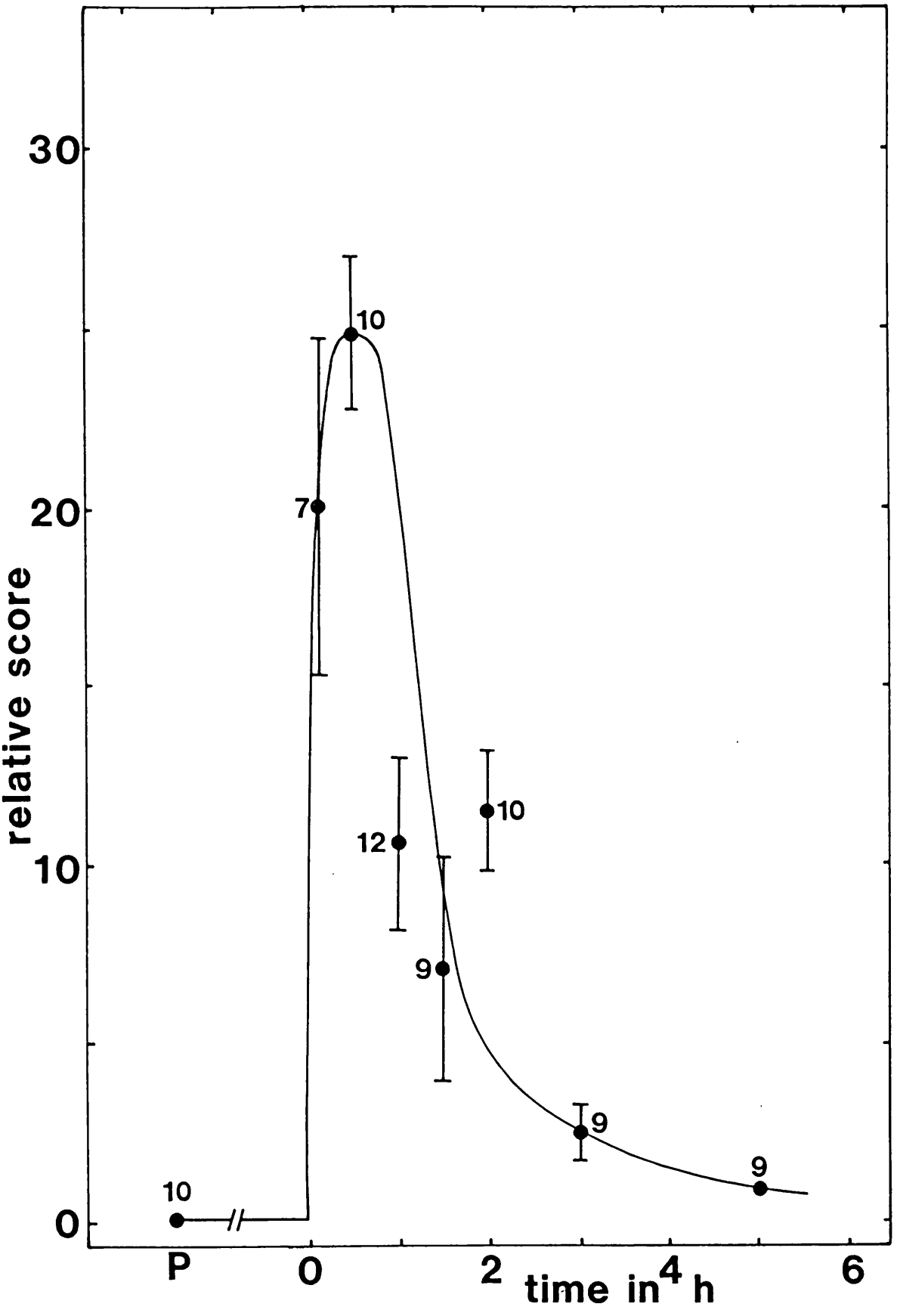

30
20
10
0
relative score
10
7
10
12
10
9
9
9
P
0
2
4
6
time in h

Fig. 10. Titres of bursicon in the ventral nerve cord during metamorphosis in *Tenebrio molitor*

TG - thoracic ganglia; AG - abdominal ganglia.

Abscissa: ph - pharate pupa (moulting fluid resorbed and therefore within a few hours of ecdysis); Ep - sampled during pupal ecdysis; Ep + 2.5 h - sampled 2.5 h after pupal ecdysis; 2 d, 4 d, 6 d - days after pupal ecdysis; Ea - 10 th - sampled 10 h before adult ecdysis; Ea - sampled during adult ecdysis; Ea + 2.5 h - sampled 2.5 h after adult ecdysis. The heights of the bars indicate the means of two separate experiments, in each of which a pooled homogenate of 20 tissues was bioassayed by injection into 10 flies to give a mean tanning score. As in Fig. 7, the relative score is computed as the actual tanning score achieved multiplied by the dilution (one quarter of a tissue was injected into each fly).

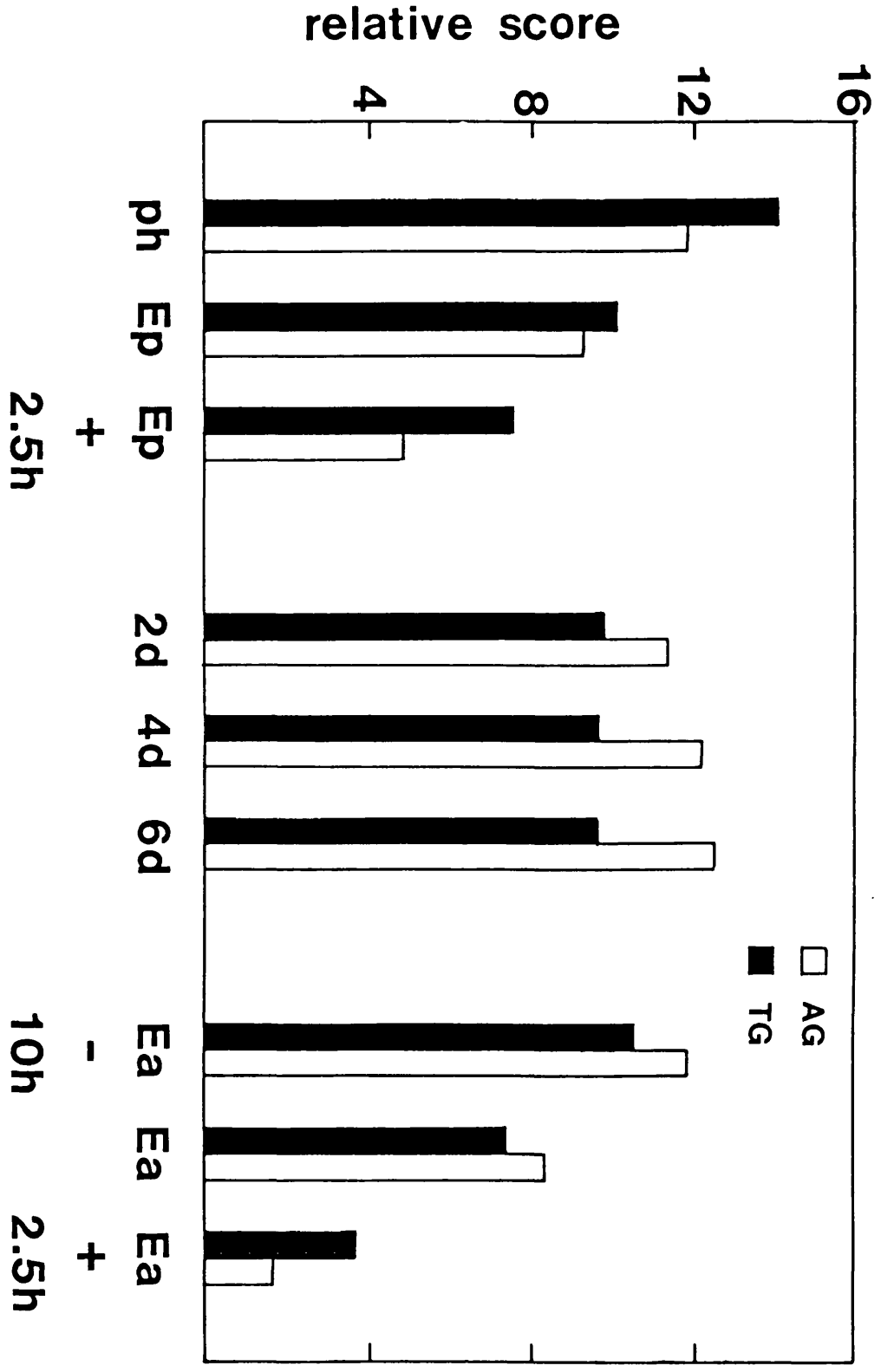

relative score
4
8
12
16
ph
Ep
Ep
2d
4d
6d
Ea
Ea
Ea
+
2.5h
-
10h
+
2.5h
AG
TG

Fig. 11. Effect of neck ligation on bursicon activity in the blood of *Tenebrio molitor* during adult ecdysis.

Neck ligation was performed 1 - 3 h after pupal ecdysis. Details as for Fig. 7 .

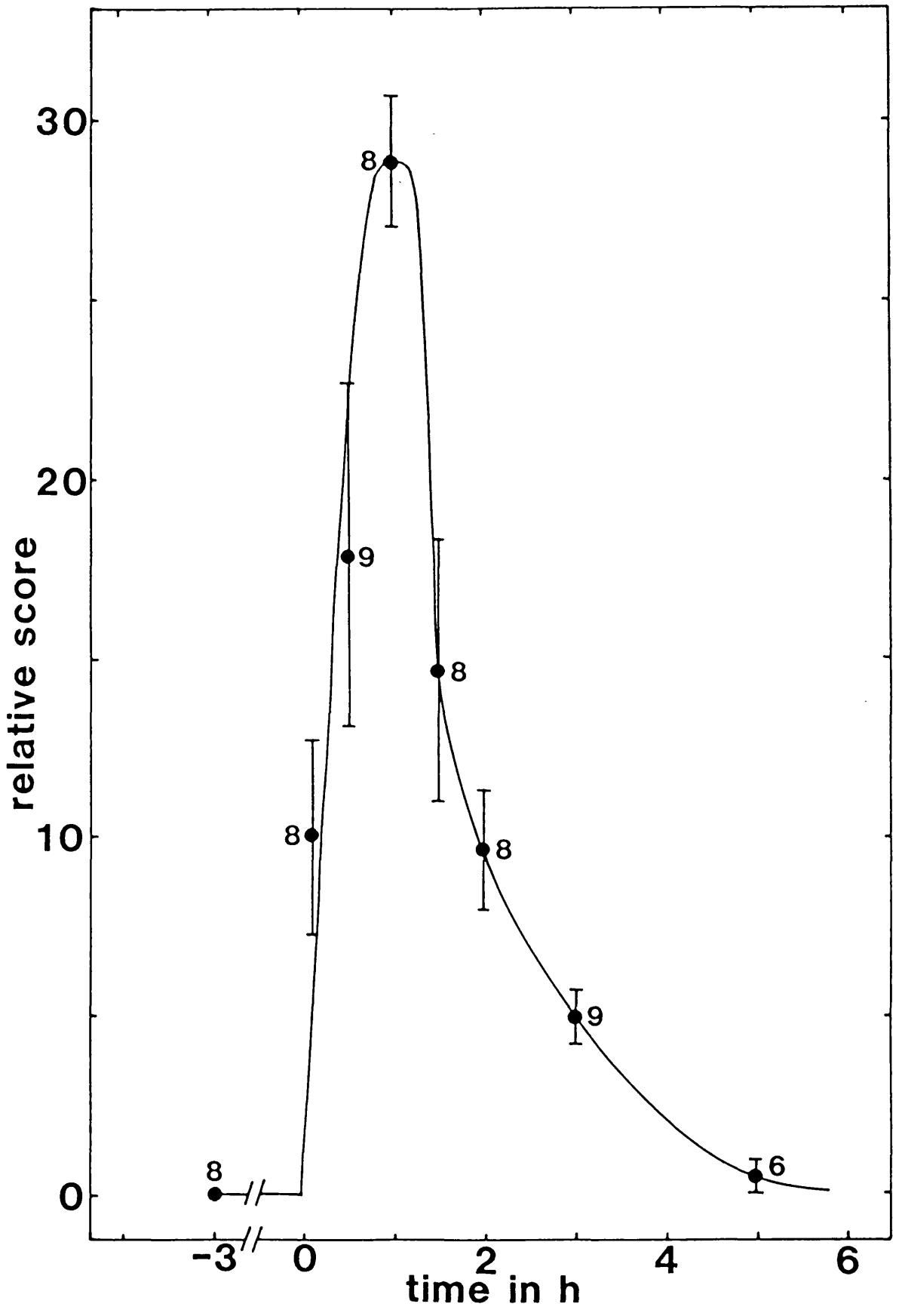

30
20
10
0
relative score
8
9
8
8
8
9
8
6
-3
0
2
4
6
time in h

Table 6. Effect of neck-ligation on bursicon activity in the blood of *Tenebrio molitor* 45 mins after the onset of ecdysial behaviour.
Neck ligations performed 24 h before the adult ecdysis.

	Mean Relative Score*	SE	N
Operated	25	1.9	9
Normal	17.8	2.7	9

* See legend to Fig. 7.

Statistical comparison of data

	t	P	
Operated v normal	2.18	0.05 - 0.02	significant

Table 7. Effect of blood transfusions on the tanning score of neck-ligated adult *T. molitor*

Neck ligation performed 1-3 h after the pupal ecdysis

Column	1	2	3		4	5		6
Treatment	Age of donor h before adult ecdysis	Age of recipient h before adult ecdysis	Tanning* score -5- point scheme Mean	SE	% Tanning+	Tanning* score,7- point scheme Mean	SE	N
Non-injected control	-	-	0.78	0.09	34	1.02	0.12	54
Injected with blood	3	3	1.58	0.13	76	1.84	0.20	40
Injected with saline	-	3	1.18	0.18	66	2.02	0.18	16
Injected with blood	12	12	1.37	0.23	76	1.68	0.10	27
Injected with saline	-	12	1.15	0.21	66	1.30	0.16	13

* insects scored 48 h after ecdysis

+ % tanning = % of insects with class 3 and class 4 scores (5-point scheme) among the total.

Table 7a Statistical comparisons of the data shown in Table 7.

Tanning score 5 -point scheme	t	P	
Age of donor & recipient = 3h			
Non-injected control v injected blood	5.06	< 0.001	highly significant
Injected saline v injected blood	1.802	0.1 - 0.05	not significant
Non-injected control v injected saline	1.988	0.1 - 0.05	not significant
Age of donor and recipient = 12h			
Injected saline v injected blood	0.707	0.5 - 0.4	not significant
Non-injected control v injected blood	2.389	0.05 - 0.02	significant
Tanning score 7-point scheme			
Age of donor and recipient = 3 h			
Non-injected control v injected blood	3.52	< 0.001	highly significant
Injected saline v injected blood	0.669	0.6 - 0.5	not significant
Non-injected control v injected saline	4.630	< 0.001	highly significant
Age of donor and recipient = 12h			
Injected saline v injected blood	2.01	0.1 - 0.05	not significant

Table 8. Effect of blood transfusion on the tanning score of neck-ligated adult *T. molitor*

Neck-ligation performed 24 h before adult ecdysis.

Treatment	Age of donor h before adult ecdysis	Age of recipient h before adult ecdysis	Tanning score* Mean	SE	N
Non-injected control	-	-	1.46	0.12	31
Injected with blood	0	1-3	1.82	0.12	36
Injected with saline	-	1-3	1.8	0.14	30

* insects scored 48 h after ecdysis

Statistical comparison of data:	t	P	
Non-injected control v injected blood	2.12	0.05	significant
injected blood v injected saline	0.108	>0.9	not significant
non-injected control v injected saline	1.844	0.1 - 0.05	not significant

Table 9. Effect of blood transfusions on the tanning score of thorax-ligated adult *T. molitor*

Thorax-ligation performed 1-3 h after the pupal ecdysis

Treatment	Age of donor h before adult ecdysis	Age of recipient h before adult ecdysis	Tanning score*		N
			Mean	SE	
Non-injected control	-	-	1.14	0.14	7
Injured+ control	-	3	1.90	0.3	8
Injected with saline	-	3	2.04	0.12	8
Injected with blood	3	3	1.46	0.1	7

* insects scored 48 h after ecdysis

+ pricked with a needle

Statistical comparisons of data

	t	P	
Non-injected control v injured control	2.296	0.05 - 0.02	significant
Non-injected control v injected saline	4.891	< 0.001	highly significant
Non-injected control v injected blood	2.971	0.02 - 0.01	significant
Injured control v injected saline	0.433	0.7 - 0.6	not significant
Injured control v injected blood	1.392	0.2 - 0.1	not significant
Injected saline v injected blood	3.777	0.01 - 0.001	highly significant

Statistical comparisons of data

	t	P	
Injured control v injected saline	1.51	0.2 - 0.1	not significant
Injured control v injected nerve cord 24h	0.805	0.5 - 0.4	not significant
Injured control v non-injected	4.10	<0.001	highly significant
Injected saline v injected nerve cord 24 h	1.932	0.1 - 0.05	not significant
Injected saline v non injected	4.468	<0.001	highly significant
Injected nerve cord 24 h v non-injected	1.789	0.1 - 0.05	not significant
Injected 6-12h nerve cord v injured controls	1.073	0.4 - 0.3	not significant
Injected 6-12 h nerve cord v non-injected controls	1.521	0.2 - 0.1	not significant

Table 10. Effect of neck cord extracts on the tanning score of neck-ligated insects

Neck-ligation performed 1-3 h after the pupal ecdysis

Treatment	Age of donor h before adult ecdysis	Age of recipient h before adult ecdysis	Tanning score* Mean	SE	N
Non-injected control	-	-	1.14	0.1	54
Injured+ control	-	3	1.72	0.1	7
Injected with saline	-	3	2.06	0.18	7
Injected with nerve cord extract	24	3	1.54	0.2	8
Injected with nerve cord extract	6-12 ∂	18	1.48	0.2	7

*insects scored 48 h after ecdysis + pricked with a needle

∂ mixture of two stages, 6 and 12 "late" (see Table 1, General Materials and Methods)

Fig. 12. The effect of neck ligation on the rate of abdominal pumping

Legend: Abscissa: time in hours before ecdysis

Ordinate: number of flexions per minute

Vertical lines denote SE of the mean

Figures denote number of determinations

Points joined by a continuous line are normal insects

histogram, insects ligated 1-3 h after pupal ecdysis

■ insects ligated 18 h before adult ecdysis

▲ insects ligated 24 h before adult ecdysis

Statistical comparisons of data

		t	P	
Normal insects	-4 h v 0.5 h	3.892	0.01 - 0.001	significant
1-3 h ligated	-12 h v -3 h	1.968	0.1 - 0.05	not significant
	-3 h v -1 h	3.498	0.01 - 0.001	significant
-18 v -24 h ligated	at -1 h	3.156	0.01 - 0.001	significant

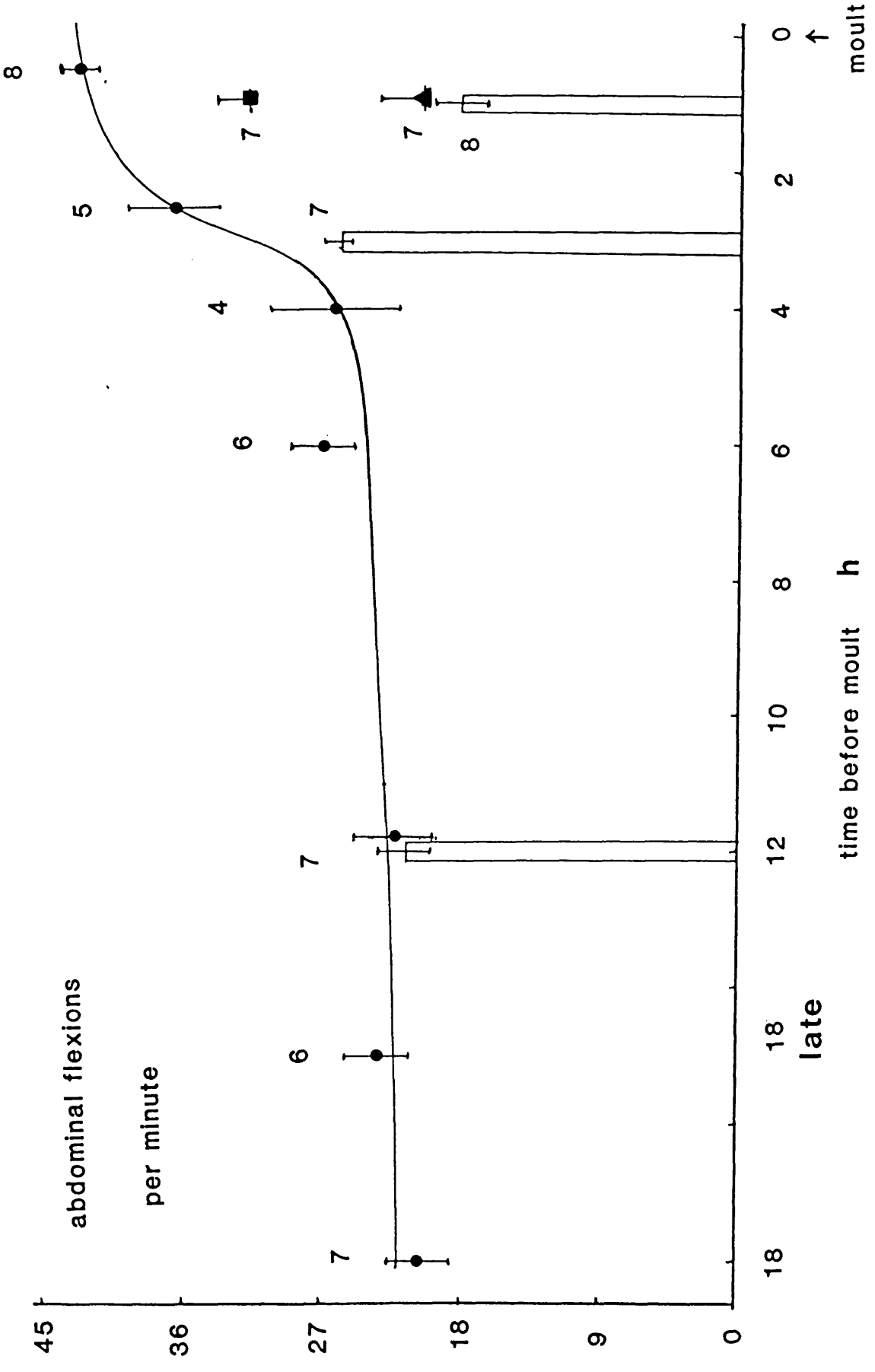

abdominal flexions
per minute
45
36
27
18
9
0
8
5
4
6
7
6
7
7
7
8
7
18
late
12
10
8
6
4
2
0
moult
time before moult h

Fig. 13. Effect of neck ligation on the ability to shed the exuvia

Legend: Abscissa; time of ligation in hours before ecdysis

Ordinate; ecdysial score (see Table 2B)

Vertical lines denote SE of the mean. Figures denote number of determinations

developmental stage at the time of ligation determined retrospectively

(see page 27)

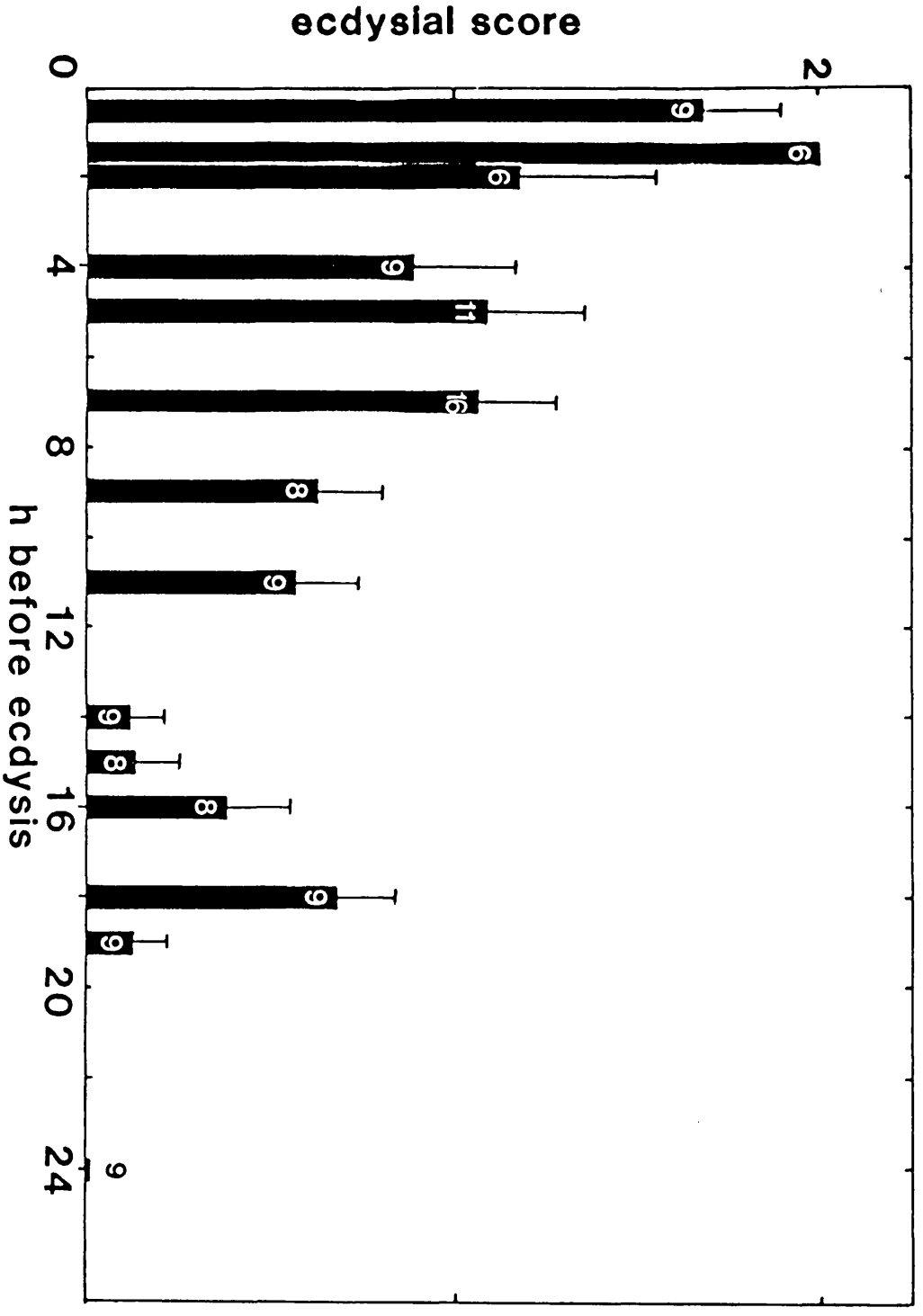

ecdysial score
0
2
4
8
12
16
20
24
h before ecdysis
9
6
6
6
11
12
8
6
6
8
8
6
6
9

Fig. 14. Pre-ecdysial plasticization of pharate adult elytral cuticle.

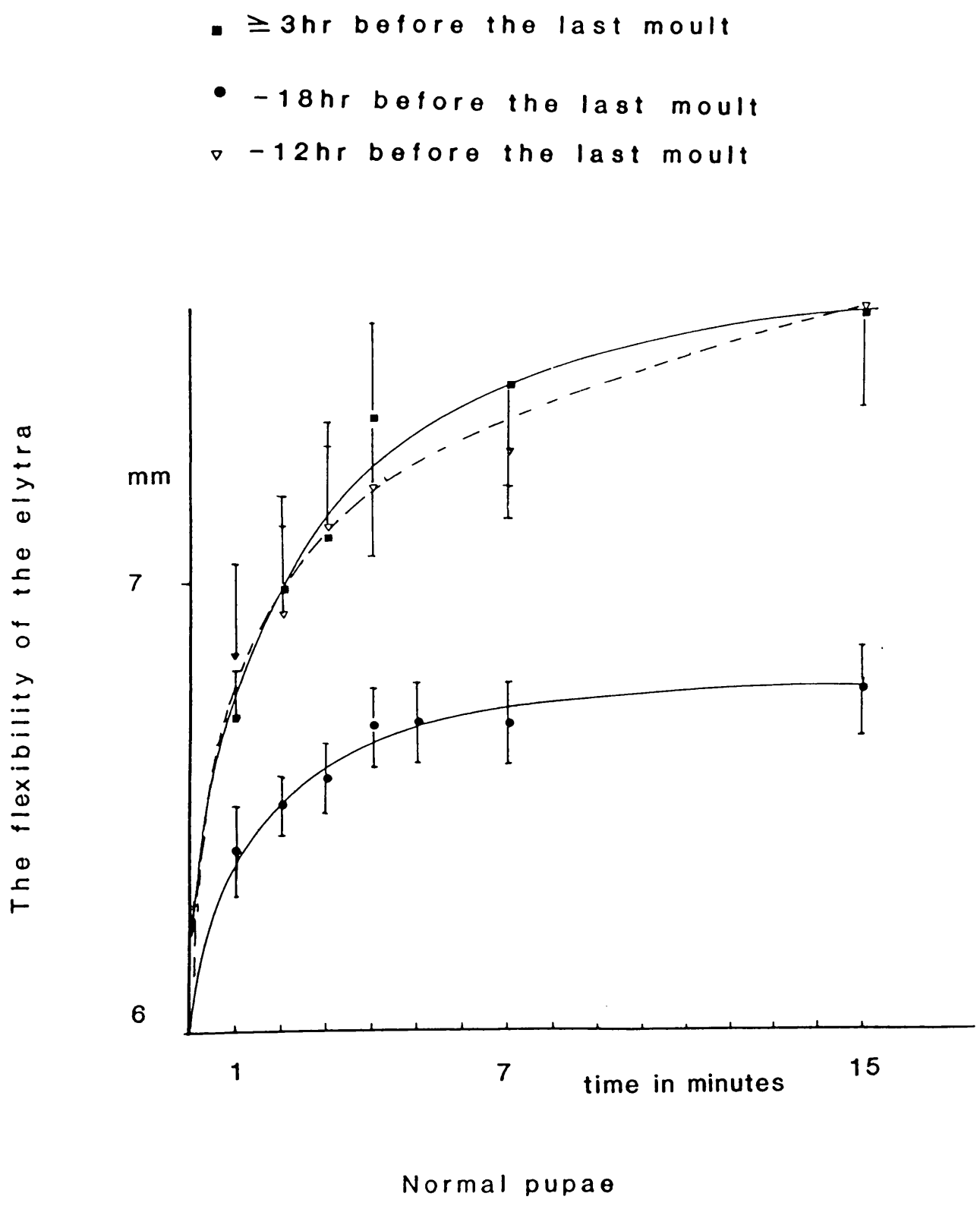
Legend: Abscissa: time in minutes after the application of the 0.3 g load

Ordinate: distance in mm between two paint marks on the elytra.

Vertical lines denote SE of the mean

Experiments performed on elytra taken from insects at different time periods prior to the last ecdysis, as indicated on the graph.

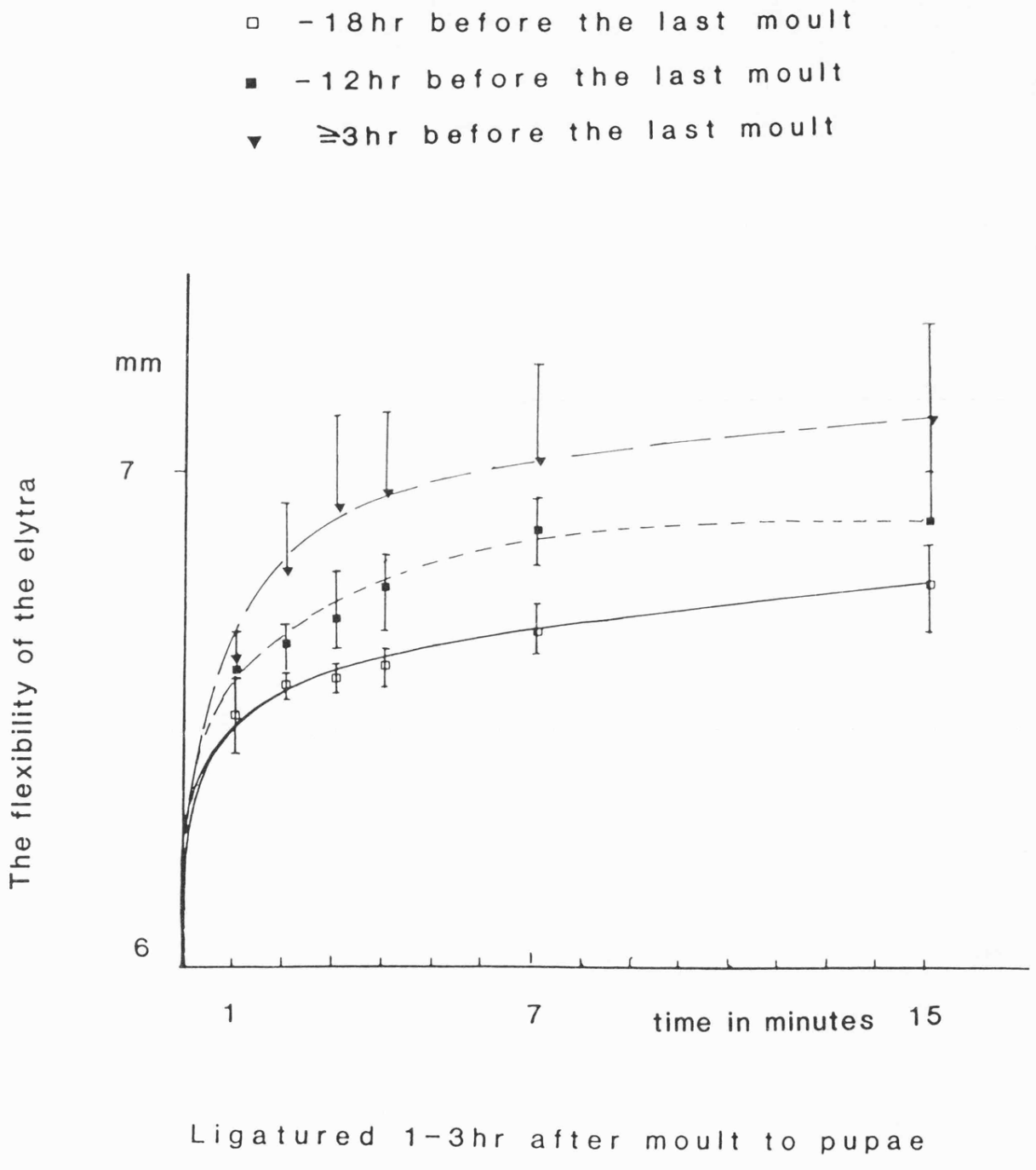
Number of determinations = 5 in each case.



Normal pupae

Fig. 15. Effect of neck ligation on the pre-ecdysial plasticization of pharate adult elytral cuticle. ligation performed 1-3 h after the pupal ecdysis.

Legend as for Fig. 14.

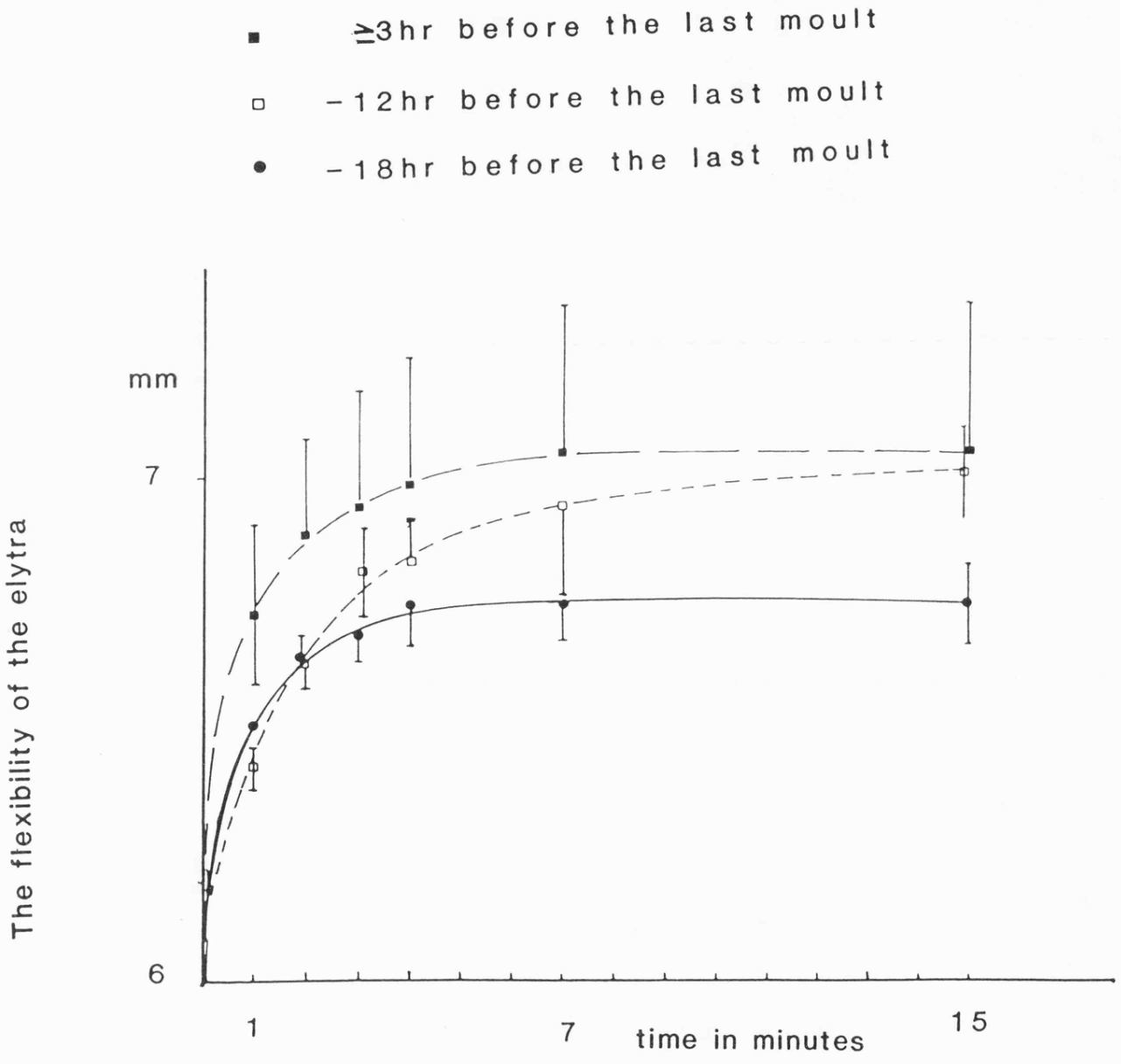


Ligatured 1-3hr after moult to pupae

Fig. 16. Effect of neck ligation on the pre-ecdysial plasticization of pharate adult elytral cuticle ligation performed 24 h before the adult ecdysis.

Legend as for Fig. 14.

At least 5 determinations in each case



Ligatured −24 hr before the last mout

Fig. 17. The effect of neck-ligation on the pre-ecdysial plasticization of the pharate adult elytral cuticle. The results depicted in Figs. 14, 15, and 16 have been re-expressed as follows: the distance between the paint marks on the elytra after 15 minutes under load are expressed as a percentage of the starting values.

Legend: Abscissa: age of the insects, from which elytra were taken, in hours before the last ecdysis

Ordinate; % extension after 15 minutes under load (0.3 g)

Vertical lines denote SE of the mean. Figures denote number of determinations. Lig 1-3 P = ligated 1-3 h after pupal ecdysis. Lig. -24 = ligated 24 h before adult ecdysis. N = normal pharate adults.

Statistical comparisons of data		t	P	
lig 1-3	-18 v -12	1.35	0.3 - 0.2	not significant
	-12 v < -3	0	> 0.9	not significant
lig -24	-18 v -12	1.05	0.4 - 0.3	not significant
	-12 v < -3	0	> 0.9	not significant
N	-18 v -12	5.31	< 0.001	highly significant
	-12 v < -3	0	>0.9	not significant

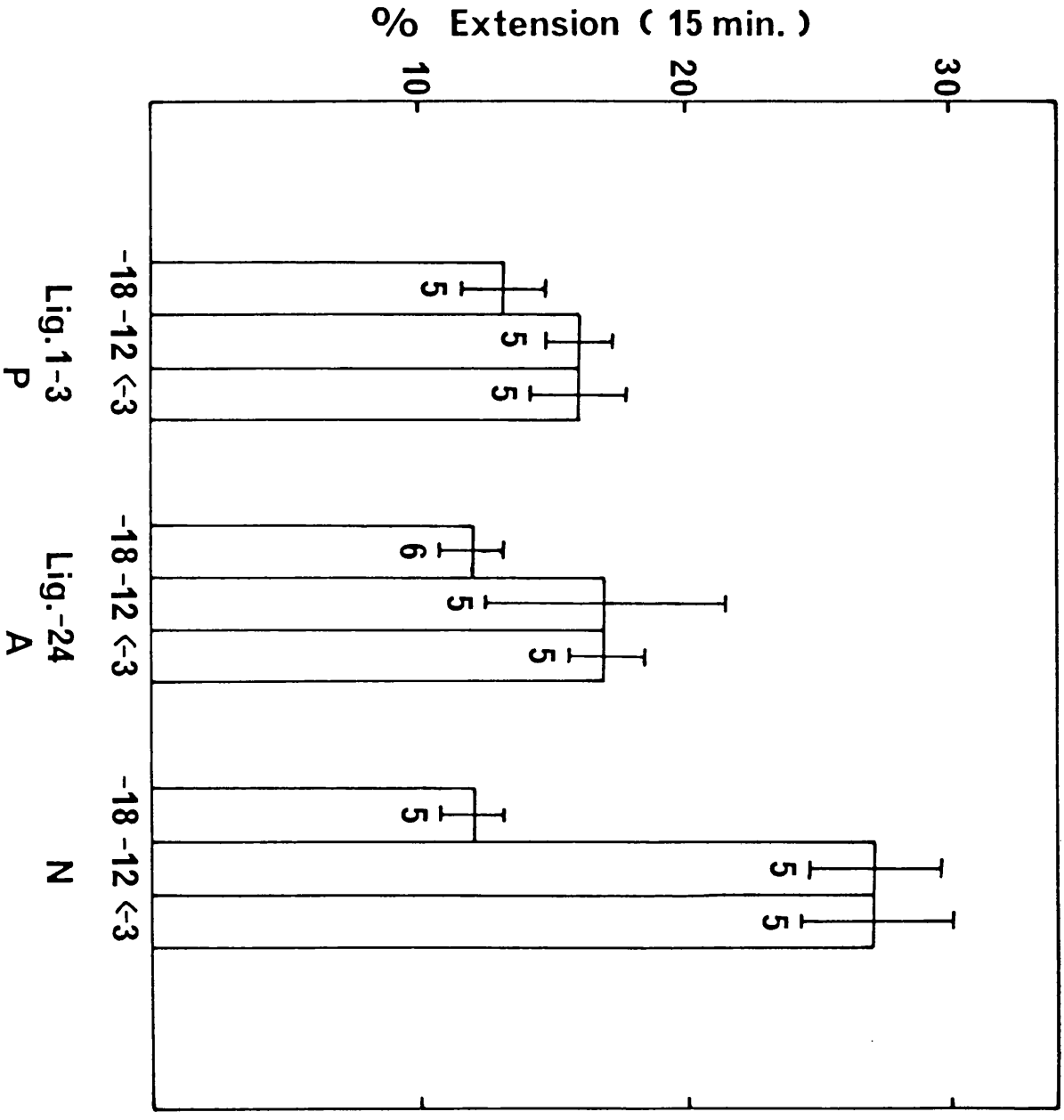

% Extension (15 min.)
10
20
30
Lig.1-3
P
-18 -12 <-3
5
5
5
Lig.-24
A
-18 -12 <-3
6
5
5
N
-18 -12 <-3
5
5
5

Fig. 18. Pre-ecdysial plasticization of pharate adult elytral cuticle

The experiment depicted in Fig. 14 was repeated but this time insects were used at more closely observed stages of development, in order to try and identify the onset of plasticization of the elytral cuticle more precisely.

Legend: Abscissa; time at which elytra were dissected, prior to ecdysis (see Table 1 Chapter 1)

Ordinate; % extension of the elytra 15 minutes after loading with 0.3 g weight.

Vertical lines denote SE of the mean

Figures denote the number of determinations

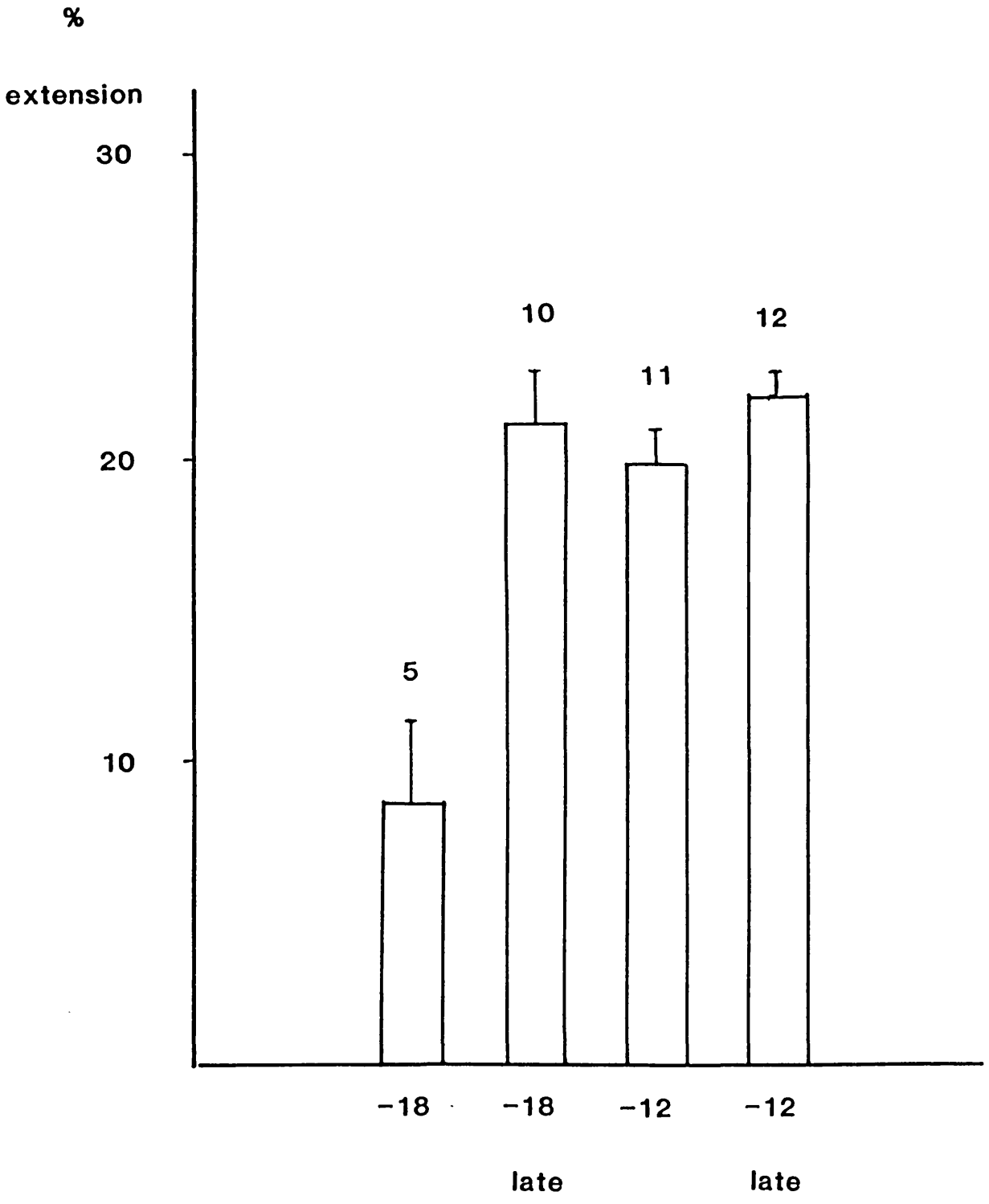

%
extension
30
20
10
5
10
11
12
-18
-18
late
-12
-12
late

Fig. 19. The effect of neck-ligation on post-ecdysial expansion of the elytra

Legend: Abscissa; time of ligation in hours before ecdysis

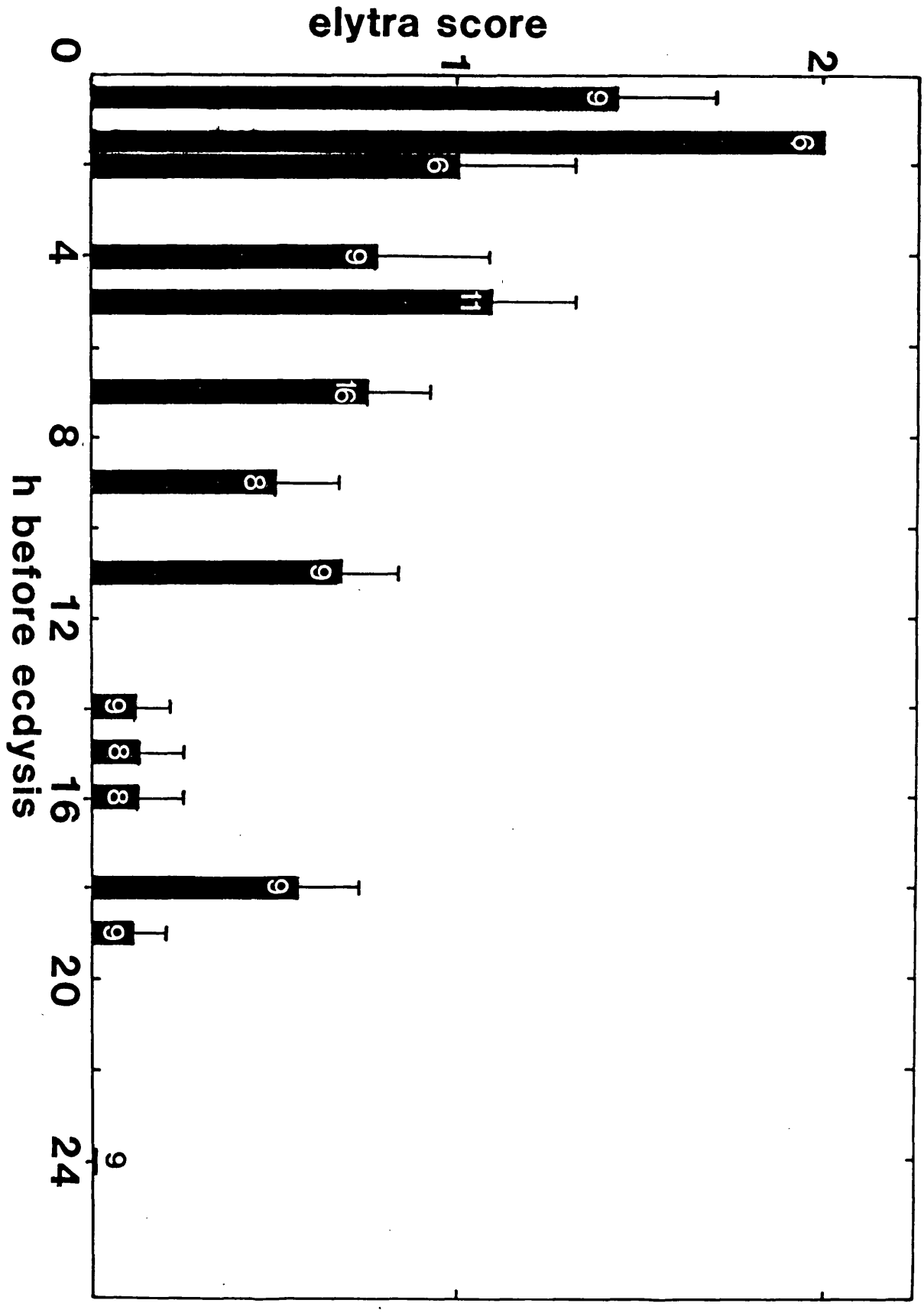
Ordinate; elytra score (see Table 2A)

Vertical lines denote SE of the mean

Figures indicate number of determinations

developmental stage at the time of ligation determined retrospectively

(see page 27)


elytra score
0
1
2
h before ecdysis
0
4
8
12
16
20
24
6
9
6
6
11
9
8
6
9
8
8
6
9
9

Discussion

The results of this study are consistent with findings in other insects (reviewed by Reynolds, 1983), in that in *Tenebrio* bursicon is released into the blood only at the time of ecdysis in the form of a brief pulse. In particular, no evidence was found for pre-ecdysial release of bursicon in *Tenebrio* as has been suggested to occur by Delachambre and his co-workers.

In agreement with Delachambre (1971) it was found that neck ligation 1 - 3 h after pupal ecdysis almost completely prevented post-ecdysial tanning. Delachambre showed that this failure to tan following otherwise normal adult development was due to the absence from the blood of a factor normally released from the PVOs of the ventral nerve cord about 2 d before adult ecdysis. Evidently this release required some unknown activating signal from the head. He was able to restore tanning in the ligated insects by injecting active blood, or extracts of the ventral nerve cord, and proposed that the active factor in these experiments was directly responsible for post-ecdysial tanning, even though this did not occur until some time after the factor was normally released. He called this factor "bursicon".

However, it seems unlikely that Delachambre's active factor is really bursicon. In every other insect examined, bursicon is released only at ecdysis, and acts rapidly to

initiate post-ecdysial tanning. In the present work evidence is presented that *Tenebrio* possesses a hormone which has all the classical characteristics of bursicon, and which is released only at ecdysis. This hormone is clearly not the same as Delachambre's factor, because (i) bursicon is not released pre-ecdysially whereas Delachambre's factor is, and (ii) bursicon is released at ecdysis even in neck-ligated insects, whereas Delachambre's factor is not.

If bursicon is responsible for initiating post-ecdysial tanning in *Tenebrio* as it is in other insects, then it follows that Delachambre's factor must affect tanning in some other way. Since it is shown here that bursicon is released normally even in neck-ligated insects which do not tan, then Delachambre's factor (DF) cannot act to promote or permit the release of bursicon. Delachambre's factor could act to promote the competence of the epidermis to respond to bursicon when this is subsequently released at the time of ecdysis. Just such an acquisition of competence occurs in *Manduca*, where the integument of the pharate adult moth is unresponsive to bursicon until a few hours before the hormone is released (Reynolds *et al.*, 1979). The proximate cause of the acquisition of bursicon responsiveness in *Manduca* is not known, although it may depend on the fall in the titre of haemolymph ecdysteroids which occurs before ecdysis, and which appears to be esential for the release of eclosion hormone (Truman, 1981) and the coordination of other developmental events which precede ecdysis (J.W. Truman, personal communication).

However, it seems unlikely that the falling titre of ecdysteroids could promote the competence of *Tenebrio* epidermis to respond to bursicon. Although thorax-ligated insects fail to tan (Delachambre, 1971; the present work), Delbecque *et al.* (1978) found that the titre and pattern of release of ecdysteroids in isolated abdomens were close to normal. In addition, the comprehensive series of experiments carried out by Delachambre and his colleagues are perhaps more consistent with the *presence* of a factor (here referred to as DF) which actively promotes tanning rather than with the *absence* of a factor permitting tanning.

The elytra of pharate adult *Tenebrio* exhibit an increase in extensibility under an imposed load around 18 h before the last ecdysis. A similar pre-ecdysial plasticization of wing and body cuticle has been shown for *Manduca sexta* (Reynolds, 1977) and *Schistocerca gregaria* (Elliott, 1981). In the latter two cases, plasticization occurred considerably closer to ecdysis than in *Tenebrio* (2 h and approx. 30 minutes respectively). The increase in wing (elytron) extensibility in both *Manduca* and *Tenebrio* is dependent on the presence of the head (Reynolds, 1977; present work). Reynolds (1977) showed that an eclosion hormone, released from the corpora cardiaca, was the inducing agent in *Manduca*. This raises the possibility that an as yet undescribed EH is responsible for pre-ecdysial plasticization of the elytra in *Tenebrio*. Certainly Truman *et al.* (1981) have established the presence of EH in insects of a number of orders including the Coleoptera (e.g. *Dytiscus)*.

Is it possible that other effects of neck-ligation reported here, including failure to respond to bursicon, are symptoms of EH deficiency? In other words is DF an eclosion hormone? When a comparison is made between the situation in *Manduca sexta* and *Tenebrio* the answer to this question appears to be that it is unlikely. Removal of the brain from developing pharate adult *Manduca sexta* drastically interferes with the subsequent ecdysis, in that ecdysial behaviour is badly organised and the event is no longer "gated" (Truman, 198[illegible]). In contrast, neck-ligated *Tenebrio* pupae attempt to ecdyse on time and their behaviour, with the exceptions that will be described later, is substantially normal. It should be noted that adult ecdysis in *Tenebrio* is not "gated", and the length of pupal life under the conditions of culture used here was 9 days. 1-3 h neck-ligated pupae ecdyse on time while -24 h ligated pharate adults ecdysise ca. 6 h late (presumably due to the non-specific trauma of the recent operation). An additional problem with the proposal that DF is an EH is that while EH in pharate adult *Manduca sexta* is released as a single pulse with a ½-life of 45 min in the blood (Reynolds *et al.*, 1979) DF is released over a period of 2 days in Delachambre's *Tenebrio* pupae (Delachambre, 1971) and probably over an 18h period in "Bath University" *Tenebrio* pupae (present work).

Hughes (1978) also failed to find direct evident of an EH in *Schistocerca gregaria;* blood transfusions from emerging adult locusts into mid-fifth instar larvae did not induce

premature ecdysis. He concluded that a suitable perch was sufficient to elicit ecdysial behaviour. However, Elliott (1981) has suggested that the existence of an eclosion hormone in the desert locust is not precluded by Hughes' observations. "In locusts, the hormone could prime rather than trigger emergence. It may be that a primitive role for the 'eclosion hormone' is to soften the cuticle; in advanced insects it may serve other functions". The former situation could apply to *Tenebrio*. Therefore the effects of neck-ligation will be discussed in the light of the hypothesis that *Tenebrio* produces an eclosion hormone.

It is difficult to appreciate the biological significance of pre-ecdysial plasticization of the elytral cuticle, unless the phenomenon is a reflection of an overall softening of the cuticle prior to ecdysis. This, as Elliott (1981) has proposed for *Schistocerca gregaria*, might allow partial expansion of the cuticle during ecdysis and allow bending of appendages around joints in the exuvia. In *Tenebrio*, the failure of neck-ligated insects to expand their elytra could be due to the absence of the period of pre-ecdysial cuticular plasticization. However, elytral expansion occurs post-ecdysially at the time of bursicon release into the blood. Thus an as yet undiscovered post-ecdysial, bursicon-dependent, period of cuticle plasticization, similar to that reported by Reynolds (1976, 1977), in *Manduca sexta* and *Calliphora vicina*, may be responsible. Bursicon could make an additional contribution to the expansion

of the elytra by inducing tanning of the veins. This could cause shrinkage due to dehydration, resulting in compression of the blood in the veins with concomittant straightening of cuticular folds and inflation of the elytra (Reynolds, 1980).

The alternative proposals for the inter-relationship between cuticle plasticization and expansion of the elytra described above are not mutually exclusive and indeed may be unified by the concept of an EH in *Tenebrio*. If neck-ligation prevents the release of EH then there would be no EH-dependent pre-ecdysial cuticle plasticization and, without an EH-dependent acquisition of competence to respond, no bursicon-dependent post-ecdysial cuticle plasticization or wing vein tanning.

Unfortunately the critical periods for the head in elytra expansion and cuticle plasticization are different (RT_{50} = - 3½h and between -18 and -24 h*respectively) as are the critical periods for the head in elytra expansion and tanning (i.e. ability to respond to bursicon) (RT_{50}s = -3½h and -11 h respectively), which militates against a common mechanism of control.

Insects neck-ligated either 1-3 h after the pupal ecdysis or 24 h before the adult ecdysis display substantially normal ecdysial behaviour (see earlier in the discussion). However, the normal increase in abdominal pumping, which occurs approx. 4 h prior to ecdysis, is prevented by neck ligation at or before

*the time period between the onset of cuticle extensibility and the last ligature (-24 h) known to prevent plasticization; thus the critical period is approximate

-24 h. Provansal *et al.* (1977) showed that abdominal pumping was responsible for the steady increase in haemolymph pressure prior to adult ecdysis, though the final large elevation in pressure which resulted in rupture of the cuticle at the dorsal suture was caused by anteriad peristalsis of the abdominal muscles.

Slama (1980) demonstrated that exogenous administration of ecdysterone inhibited ecdysis in pupae of *Tenebrio molitor* and reduced haemolymph pressure when injected 5 - 10 h prior to ecdysis. However, the reduced rate of pumping in ligated pupae of *Tenebrio* cannot be accounted for on the basis of elevated titres of ecdysterone, as the pattern of release of ecdysterone in isolated abdomens is not significantly different from that in normal insects (Delbecque *et al.*, 1978). Provansal *et al.* (1977) suggested that the neuromuscular events which caused the elevated haemolymph pressure at ecdysis were under the control of a neurohormone. It is tempting to suggest that DF is that hormone. The critical period for the head in tanning (RT_{50} = -11 h) was of the same order as that for abdominal pumping (not determined precisely but -18 h ligated insects exhibited an elevated rate of pumping with respect to -24 h ligated insects, approx RT_{50} = -18 h).

The consequences of the lack of elevated rate of abdominal pumping and hence presumably lack of initial increase in haemolymph pressure are not immediately apparent as the

pronotal cuticle still splits along the dorsal suture (personal observation). However, insects neck-ligated prior to -24 h fail to shed the exuvia. It is conceivable that the last named is a direct result of a reduced haemolymph pressure, though the critical period for the head in shedding of the exuvia, RT_{50} = - 4h, would appear to be substantially later than that for abdominal pumping (see earlier).

Post-ecdysial bursicon-mediated plasticization of the cuticle (see above), if such occurs in *Tenebrio*, could also contribute to the act of shedding the cuticle. Elliott (1981) pointed out that softening of the new cuticle in the desert locust might aid bending of appendages around joints.

If post-ecdysial cuticle plasticization does not occur in neck-ligated *Tenebrio*, due to a failure to respond to bursicon in the absence of the eclosion hormone, a significantly reduced ability to shed the exuvia may result. Unfortunately there is a lack of concordance of the requisite critical periods for the head (cp) viz. RT_{50} in tanning (or more correctly in ability to respond to bursicon) is -11 h in comparison to -4 h for shedding of the exuvia.

Failure to shed the exuvia and expand the elytra could of course simply be due to the failure of ligated insects to swallow air. In many insects air swallowing occurs during ecdysis and contributes to the increase in haemolymph pressure

necessary for splitting the exuvia e.g. *Locusta migratoria* (Bernays, 1972), *Teleogryllus oceanicus* (Carlson and Bentley, 1977). However, many insects do not swallow air until after the cuticle is split to aid expansion of the new cuticle e.g. adult *Calliphora vicina* (Cottrell, 1962c), adult *Pieris brassicae* (Cottrell, 1964). The same is true of *Tenebrio* (unpublished observations, based on dissections). Since insects neck-ligated at -1 h shed the exuvia and expand the elytra, air swallowing cannot be important for these events.

Failure to shed the old cuticle could also be due to a lack of synchrony between resorption of the moulting fluid and ecdysis. In *Manduca sexta* if resorption of the moulting fluid has progressed too far then the dry exuvia tears during shedding and appendages remain encased in exuvia (Truman, 1980). This does not appear to be a problem for 1-3 h ligated pharate adult *Tenebrio* as moulting fluid resorption starts on time (personal observation).

An attempt has been made above to discuss the effects of neck-ligation solely in terms of an eclosion hormone. However, it is clear that the critical periods for the head in the phenomena described are not identical and as such do not lend support for a common mechanism of control. An alternative approach is to group the phenomena affected by the operation on the basis of their critical periods viz. (1) shedding of the exuvia (RT_{50} = -4 h) and expansion of the elytra (RT_{50} = -3½h); (2) ability to respond to bursicon (RT_{50} = -11 h), elytral

cuticle plasticization (approx. critical period between - 18 h and - 24 h), abdominal pumping (approx. RT_{50} = -18 h).

The hypothetical EH can perhaps be retained to account for group 2. However, on the basis of critical periods, group 1 would appear to be controlled separately. Failure to shed the exuvia could influence directly the ability of a ligated insect to expand its elytra, thus leaving only 1 unexplained event.

In conclusion, evidence is provided here for the existence of a classical bursicon hormone in *Tenebrio molitor,* released in large quantities over a short period after both pupal and adult ecdyses. The hypothesis is made that Delachambre's factor (here referred to as DF) is not bursicon but another prior endocrine event which promotes competence to respond to bursicon. However, at variance with the findings of Delachambre (1971), Grillot *et al.* (1976) and Delachambre *et al.* (1979), ligation and replacement therapy experiments failed to demonstrate the presence of a factor in blood and nerve cord which promotes tanning. In addition, the critical period for the head in tanning was considerably later in " Bath University " *Tenebrio* than in those used by Delachambre (1971). The effects of neck-ligation on other events are consistent with the hypothesis that an eclosion hormone is produced by *Tenebrio* and it could be synonymous with DF. The caveat is made that the critical periods for the head are dissimilar. This could at least in part be due to

the fact that, of necessity, the staging of development of insects was more precise in some experiments than others (see general materials and methods). However, an addition, interpretation, based solely on a comparison of critical periods, is that elytral cuticle plasticization, abdominal pumping and ability to respond to bursicon are controlled by a hypothetical EH, but the effects of neck-ligation on shedding of the exuvia and expansion of the elytra require alternative explanation.

CHAPTER 3

THE CONTROL OF DIGESTIVE ENZYME SECRETION IN *TENEBRIO MOLITOR*

Introduction

It has been said that the production of digestive enzymes is probably a continuous process in many insects since the alimentary canal especially in larvae is often kept full of food, except at the moult (Waterhouse, 1957). It has further been suggested that therefore control of secretion is only necessary in intermittent feeders. However, a sharp distinction between automatic secretion in frequent feeders and discontinuous secretion in occasional feeders cannot be sustained, all insects tend to show an increase in enzyme activity after feeding (Dadd, 1970), and secretory control may be expected to occur in most insects.

The midgut is the principal digestive organ, it secretes most of the digestive enzymes and absorbs the products of digestion (Wigglesworth, 1972). Since most enzyme activity is found in the midgut contents, it is suggested that the synthesis of enzyme within the epithelium and release of digestive fluid are interdependent processes (e.g. amylase in *Prodenia punctata* (Applebaum *et al.*, 1963); protease in *Tenebrio molitor* (Dadd, 1956)). Langley (1966) also found no protease in the midgut tissue of *Glossina morsitans* and suggested as an alternative

that an inactive zymogen occurred in the tissues, requiring activation by a kinase from elsewhere. However, to date no such trypsinogen/trypsin like activation has been demonstrated in insects. In contrast to continuous feeders at least some occasional feeders accumulate substantial intracellular enzyme activity during starvation, at which time the gut lumen is devoid of enzyme, to be discharged on feeding, *Dytiscus marginalis* (Dadd, 1956); *Stomoxys calcitrans* (Lehane, 1976). In *Aedes aegypti*, when a blood meal has been digested, the gut is cleared of proteolytic enzymes by excretion (Briegel, 1975). This does not happen in frequent feeders where substantial enzyme activity occurs in the midgut whether fed or not (Dadd, 1970).

Three possible mechanisms have been suggested for the stimulation of secretion of digestive enzymes in insects: (a), Nervous, the act of feeding, the detection of food or the presence of food may set up a nerve reflex to which secretory cells respond ; (b) Secretogogue, the foodstuff or its products stimulate secretion; (c) Hormonal, like the nervous mechanism except that the feeding results in the production of a hormone that reaches the digestive tract via the haemolymph (House, 1974).

The possibility of a direct nervous stimulus does not appear to have been seriously considered since Day and Powning (1949) observed that the nerves innervating the midgut of *Blatella germanica* appear to be motor and to supply only the musculature. Although their evidence was purely anatomical,

the time lag between feeding and increased enzyme levels also militates against nervous regulation. In spite of this more recent studies suggest that the latter cannot be completely ruled out. Geering and Freyvogel (1975) found that the stretching of the gut of *Aedes aegypti* by air enemas resulted in an increase in non specific esterase production although triglyceride lipase was not affected. Dadd (1956) observed a very rapid discharge of protease within minutes of *Dytiscus marginalis* pouncing on its prey. The results of both these studies are best explained on the basis of nervous regulation.

Many authors have cited that the level of proteolytic enzymes in the gut lumen increases after food ingestion, in various species of Diptera (Fisk, 1950; Fisk and Shambaugh, 1952; Yang and Davies, 1968), in *Tenebrio molitor* (Dadd, 1956), *Periplaneta americana* (Schlottke, 1936) and *Locusta migratoria* (Khan, 1963). A qualitative or quantitative relationship has been shown between the two processes but it is difficult to establish whether the effect of feeding is direct (secretogogue) or indirect (endocrine).

Evidence for a secretogogue mechanism comes from various sources. In adult *Leucophaea maderae* high proteolytic activity is primarily found in the posterior region of the midgut lumen. Only feeding on certain types of protein, e.g. glutenin, stimulated protease activity; several other proteins, amino acids and starch did not cause increased enzyme activity. There

was also a correlation between the quantity of certain proteins passed into the midgut and the titre of protease activity found there (Engelmann, 1969). Blood but not sugar has been found to increase protease in *Aedes aegypti* (Fisk, 1950; Fisk and Shambaugh, 1952), *Stomoyxs calcitrans* (Champlain and Fisk, 1956), *Culex fatigans* (Gooding, 1966), *Glossina morsitans* (Langley, 1966) and several Simuliidae (Yang and Davies, 1968). If in the references cited sucrase had also been elicited by sucrose alone, then it could have been concluded that there was substrate specific differential secretion (absence of parallel stimulation of different enzymes by one nutrient substrate). However, in blackflies sucrose failed to raise the level of sucrase and blood increased activities of both enzyme species (Yang and Davies, 1968). In *Aedes aegypti* the stimulus for midgut enzyme secretion is dependent on the routing of food within the gut. Blood meals proceed direct to the midgut in blood-sucking flies, whereas sucrose solutions are stored in the diverticula for later gradual release and are therefore unlikely to have a direct effect on enzyme secretion (Fisk and Shambaugh, 1954). Substrate-specific secretion has recently been demonstrated in larvae of *Drosophila melanogaster* (Hosbach *et al.*, 1972) and *Attagenus megatoma* (Baker, 1977). When starved larvae of *Attagenus megatoma* were fed selected diets, increases in proteolytic, trypsin and chymotrypsin activity were correlated with total midgut protein and not with the amount of food consumed. Only a small increase in protease occurred in starch-fed larvae, yet the same larvae had the highest sucrase activity.

In addition sucrase activity in larvae fed diets that contained casein was always significantly greater when the diets also contained sucrose (Baker, 1977). The results of this study suggest that digestive enzyme release in *Attagenus* is nutrient controlled and class specific.

Studies on *Rhodnius prolixus* have also shown a strong correlation between protease activity in the midgut and the protein content of midgut homogenates (Garcia and Garcia, 1977) Blood can be substituted by egg albumen and B.S.A. but not by maltose and dextrose. Clearly abdominal distension alone is not responsible for protease secretion.

In some of the early studies on digestion in mosquitoes, e.g. Shambaugh, 1954, it is not clear whether all blood substitutes were ingested equally. This is important because the supposed substrate effects recorded, which lend support to the secretogogue theory of digestive enzyme control, might really have been volume effects (Dadd, 1970). The problem arises from an inability to introduce non-blood nutrient solutions into the midgut in equal amounts using the membrane feeding technique. Briegel and Lea (1975) have overcome this problem by administering measured solutions by enema into *Aedes aegypti*. Using this technique they showed that it was increasing protein concentration in the "meal" and not an increase in its volume that enhanced trypsin secretion. Further evidence in support of secretogogue control was provided by the fact that

globular proteins with a minimal molecular weight had to be present for protease secretion.

Despite the body of evidence there is supporting the secretogogue theory, the means by which control is exerted is still not known for any insect. However, Lehane (1977) constructed a hypothesis of the means by which secretogogue control is exerted in *Stomoxys calcitrans*. A secretogogue control of protease secretion in this insect was proposed because there was a significant correlation between midgut proteolytic activity and posterior midgut protein. No such correlation was found between posterior midgut proteolytic activity and meal weight and/or TOTAL midgut protein, as would have been expected if there were a hormonal or nervous reflex responding to the amount of food passing stretch receptors in the foregut. The midgut production cells of unfed *Stomoxys calcitrans* build a store of digestive enzymes to a point where the cells' storage systems are fully loaded. This store is released immediately after a blood meal is taken (Lehane, 1976). Lehane reasoned that the cells of the gut wall could monitor the levels of amino acids released by the actions of the first batch of proteolytic enzymes and used this information (which is a measure of the quantity of protein in the digestive part of the gut) to control enzyme production levels (Lehane, 1977). He went on to propose that the digestive enzymes are produced in direct proportion to the quantities of amino acids available for synthesis. Therefore, as the digestion of the meal is

gradually completed less amino acids will be available for enzyme synthesis and consequently less digestive enzymes will be produced.

Although very plausible the general applicability of this hypothesis must be in doubt. It should follow that amino acids are elicitors of protease activity, however, this is clearly not so in *Leucophaea maderae* (Engelmann, 1969); and protein hydrolysates have little effect on *Aedes aegypti* (Briegel and Lea, 1975). In addition certain of the proteins that stimulate protease activity in *Leucophaea maderae* are not digested by this insect.

Additional evidence in support of the secretogogue theory comes from those studies in which the removal of endocrine glands has no effect on levels of digestive enzymes. Ablation of the brain medial neurosecretory cells of blood-fed mosquitoes had no effect on midgut protease activity (Foster, 1972; Lea, 1967). A similar operation performed on *Blatta orientalis* did not significantly affect the total activity of the insect's midgut proteases (Gordon, 1970). Engelman and Wilkens (1969) removed the medial neurosecretory cells from *Sarcophaga bullata* and found that food intake dropped by half, but the amount of protease activity was proportional to the liver juice injested in both operated and operated control groups.

The first indications of a hormonal influence on digestion

were the observations by Wigglesworth (1936, 1948) that the stimulation of egg development in *Rhodnius prolixus* by the corpora allata was followed by a more rapid digestion of the intestinal contents. More direct evidence has come from the work of Okasha (1964, 1968). He found that decapitated *Rhodnius* larvae had lower intestinal protease activity than intact animals, and concluded that the neurosecretory system influenced protease synthesis. However, decapitated females retained relatively large amounts of blood in their crops for as long as three weeks after the operation (Okasha, 1968). A similar phenomenon was observed by Persaud and Davey (1971), who concluded that it was therefore wrong to interprete decreased protease levels following decapitation as due to a loss of humoral control. Neither the removal of the medial neurosecretory cells nor allatectomy eliminated the cycle of protease activity (Persaud and Davey, 1971), and protease activity was highest when the amount of food in the midgut was maximal. They suggested that the evidence contributed support for a local control of protease activity, based on a secretogogue mechanism. Recent work by Garcia and Garcia (1977) provides more direct evidence to support this proposal (see earlier in this introduction). The apparent close relationship between oogenesis in the female and protease activity, may simply prove to be an acceleration of movement of food through the gut brought about by a nervous mechanism linked to the act of mating (Persaud and Davey, 1971). A correlation between egg size in the ovaries and enzyme activity in the midgut has also been shown for *Nauphoeta cinerea*

(Rao and Fisk, 1965), but there is no proof of an endocrine involvement.

Neurosecretory control, at least in part, of protease production has been postulated for *Calliphora vicina* (Thomsen and Moller, 1963). Ablation of the medial neurosecretory cells caused a reduction of the enzyme activity in the midgut. Implantation of corpora cardiaca-allata(presumarly containing mnc hormone) into female flies deprived of mnc raised their protease activity significantly. Similarly Strangways-Dixon (1961) found that in *Calliphora vicina* the mnc are necessary for ingestion and digestion of protein.

The interpretation of the effects of endocrine gland removal on digestion are complicated by the fact that operations may interfere with enzyme activity indirectly; operated animals may not eat as much as controls; or food may be released from the crop at a subnormal rate leading to a reduced enzyme production (Engelmann, 1969; Persaud and Davey, 1971; Engelmann and Wilkens, 1969). Thomsen and Moller (1963) did not measure food consumption of their experimental flies and therefore their work cannot be taken as conclusive proof of a *direct* hormonal control of digestive enzyme secretion in *Calliphora vicina*. Muraleedharan and Prabhu (1979) found that removal of the mnc resulted in a reduction in midgut protease and invertase activities in *Dysdercus cingulatus*. However, the operated animals consumed less food than the controls. Implanting mnc

into operated animals brought about an increase in food consumption and midgut enzyme. They interpreted their results as follows: mnc stimulated feeding (either directly or indirectly by an effect on metabolism), food in turn caused a stimulation of enzyme secretion. They discounted a direct effect of mnc on enzyme secretion because an increase in enzyme secretion only occurred in animals fed protein and not sugar, following the introduction of mnc into operated insects.

It is apparent from the work reviewed above that there is doubt whether the endocrine system has some influence on the synthesis of digestive enzymes in insects. However, such an effect cannot be discounted. Briegel and Lea (1979) found that the tryptic activity of midgut homogenates from female *Aedes aegypti* was reduced in mnc ablated or ovariectomized insects.

The enzyme activity was restored to the level of unoperated controls by either reimplantation or injection of 20-hydroxy-ecdysone in a large, single dose shortly before the blood meal. This is particularly interesting as ecdysone has been detected in the adult mosquito and has been shown to increase significantly in the blood fed female (Fuchs and Schlaeger, 1973). The ovary is reportedly the source of this hormone (Hagedorn *et al.*, 1975). If ecdysteroids are directly involved in enzyme secretion mnc ablation may have its effect by removing the source of a neurosecretory factor controlling their synthesis (Briegel and Lea, 1979).

Since measured amounts of blood were given to the mosquitoes by enema the results of Briegel and Lea cannot be due to the effect of surgery on food intake or gut movement. Although the neurosecretory system and ovaries were required for a substantial part of the tryptic activity normally found in the posterior midgut after a blood meal, this endocrine-dependent activity apparently represented excess secretion of enzyme, as the rate of protein digestion was the same in operated and control animals.

Some of the supposed evidence against the involvement of the neuroendocrine system in the secretion of digestive enzymes can itself be the subject of criticism. Engelmann and Wilkens (1969) found no evidence in *Sarcophaga bullata* for neuroendocrine involvement when determinations were made between 5 and 8 hr after protein feeding. However as Briegel and Lea (1979) pointed out, this may have been too early to detect an effect, because in *Aedes aegypti*, mnc-ablated and control females were not different for the first 9 h, while by 16 h they were significantly different. Engelmann (1966) starved newly moulted female *Leucophaea maderae* for 4 - 5 days and then fed them for 1 h. Removal of the brain, corpora cariaca and corpora allata from such animals had no effect on the activity of protease in the gut 24 h after the operation. However, Dogra and Gillott (1971) have pointed out that feeding of previously starved insects brings about a massive, rapid release of neurosecretion. Therefore, if the amount of protease activity were dependent on the level of hormone circulating in the haemolymph, the

failure of the operation described above to reduce enzyme activity may be due to the fact that 24 h is insufficient time to allow for degradation of hormone circulating at the time of the operation (Gillott *et al.*, 1970).

Both Thomsen and Moller (1963) and Rao and Fisk (1965) suggested that the apparent control exerted by the medial neurosecretory cells in *Calliphora vicina* and *Nauphoeta cinerea* on midgut and ovarian development were just two examples of a general control on protein synthesis exerted by these cells. Indeed recent work has shown that neurosecretory factors do stimulate blood protein synthesis in the fat body (Steele, 1976). It may be that endocrine involvement in digestion is primarily the control of enzyme synthesis and that release is effected by secretogogues. The fact that: 1. Injection of 20-hydroxy-ecdysone into sugar-fed female *Aedes aegypti* did not stimulate tryptic activity (Briegel and Lea, 1979) and 2. The introduction of mnc into ablated *Dysdercus cingulatus* only resulted in an increase in enzyme secretion when animals were fed protein and not when fed on sugar alone (Muraleedharan and Prabhu, 1979) lends support to this hypothesis.

Some of the most convincing evidence for the involvement of hormones in the control of enzyme secretion comes from the studies on newly emerged adult insects. Langley (1966) found a linear relationship between midgut protease levels and the

weight of the blood meal ingested by recently emerged *Glossina morsitans*, no such correlation was found between midgut protease activity and the protein content of the meal. Subsequently, Langley (1967) showed that puncturing the ptilinum of *Glossina* at the time of emergence prevented expansion of the crop and the rise of protease in the midgut during the first 24 h after emergence. A hypothesis was proposed to account for these observations (Langley, 1966). The size of the meal was monitored by stretch receptors on the crop duct, nerve impulses passed along the oesophageal nerves to the neuroendocrine system causing the release of a hormone(s). The latter stimulated the production of an enzyme precursor which was then activated in the lumen of the gut by some factor in the blood serum.

Khan (1964) recorded that newly moulted starved adult *Locusta migratoria* showed a spontaneous increase in invertase activity in caeca and midgut. If the insects were neck ligatured immediately after emergence or 24 h afterwards, invertase activity was only half that of the controls. However, if 48 h elapsed before the operation was performed, the ligatured insects were no different from the controls. This endogenous response suggested control by some endocrine mechanism.

Day and Powning (1949) also adduced evidence to support the view that humoral regulation might occur in some insects. Feeding caused an increase in both enzyme activity and the rate of mitosis in the midgut of *Periplaneta americana* and *Blatella germanica*,

and they inferred that increased mitosis might be taken as an index of secretory activity. Further they demonstrated that the injection of haemolymph from fed into starved *Tenebrio molitor* accelerated the rate of mitosis. This was taken a step further by Dadd (1961) who showed that the midgut protease activity of starved adult *Tenebrio* injected with haemolymph from recently fed adults was significantly greater than controls treated with the haemolymph of starved insects. A contribution to secretory control from secretogogues seemed to be ruled out as ingestion of water, cellulose powder or flour elicited an increase in amylase and protease in starved adults (Dadd, 1956).

Newly moulted adult *Tenebrio molitor,* like those of *Locusta migratoria* exhibited a spontaneous increase in activity of a protease digestive enzyme. However, protease activity failed to develop in adults decapitated 1 day before emergence. Development occurred if the operation was performed following emergence, but at a slower rate and to a lower maximal level than in the normal adult. Preliminary work indicated that injection of the haemolymph of newly emerged adults could to some extent rehabilitate the development of enzyme inhibited by pupal decapitation. Dadd (1961) concluded that one of the hormones regulating metamorphosis and adult maturation was also responsible for controlling the development of digestive enzyme activity in the newly moulted insect. The source of this hormone may prove to be the mnc as Mordue (1966) found that cauterising these cells prior to the adult ecdysis had

a similar effect on protease activity to decapitation.

Dadd (1961) followed the development of midgut protease activity in insects that had been ligatured or decapitated 1 day pre-emergence or 1,2 or 3 days post-emergence. He found that the earlier the operation was performed the lower the resulting enzyme titre. Recently Jankovic-Hladni *et al.* (1978) reported effects of ligaturing newly moulted adult *Tenebrio* on midgut amylase activity that do not conform to the pattern observed by Dadd (1961). Insects ligated either 0 - 1 hours or 18 - 20 hours after the adult moult exhibited significantly lower amylase activities than comparable controls, when assayed 3 days after the operation. However, the older group of animals (18 - 20 hours) had only half the activity in the younger insects (0 - 1 hours), the reverse of that expected on the basis of Dadd's findings.

Abboud (1981) attempted to shed light on these apparently conflicting observations. His experiments revealed a considerably more complex system of control than that envisaged by Dadd (1961) or Jankovic-Hladni *et al.* (1978). The effects of timed decapitation and ablation of the pars intercerebralis on midgut and α-glucosidase activity four days after the adult ecdysis were interpreted in terms of three hypothetical factors (see Table 1). Abboud (1981) did not determine the nature of these factors. Therefore the object of the present investigation was to confirm the existence of Factor 1 and to attempt identification.

Table 1. Hypothetical factors responsible for the control of the spontaneous increase in α-glucosidase activity in the midgut of young adult beetles (from Abboud (1981)).

"Hormones"	Time of release in hours before (-) or after (+) the adult ecdysis	Mode of Action
Factor 1	RT_{50} = -3.8/9	stimulates the epithelium to secrete enzymes (indirect influence on factors 2 and 3?)
Factor 2	RT_{50} = +4.3	depresses enzyme release (allows metamorphosis of the midgut enterocytes?)
Factor 3	+18 - +24	stimulates the (new adult?) enterocytes to secrete enzymes

* RT_{50} = time of the operation which resulted in a 50% reduction in enzyme activity.

Materials and Methods

The animals employed, the staging of development and treatment of experimental animals, application of ligatures and transfusion of blood, were as described in the general materials and methods section (Chapter 1). All reagents used were AnalaR grade or the purest available and supplied by British Drug Houses or Sigma.

Preparation of gut homogenates for enzyme assay

The insect was decapitated and then placed ventral surface uppermost before elytra and membrane wings were removed. Curved fine-forceps were used to hold the insect firmly, embracing the junction between thorax and abdomen. A second pair of forceps was used to pull the thorax anteriorly, severing its connection with the abdomen and exposing the gut. The thorax with gut attached was pulled away from the abdomen until the whole of the midgut was visible. If required the rest of the gut was exposed by further incisions into thorax and abdomen. As appropriate the gut was divided up into regions (foregut,midgut, hindgut (Plate 1)and the tissue and contents from each washed quantitatively into separate Potter Elvehjem homogenisers with 1.5 ml of distilled water. Homogenisation was carried out with a Teflon pestle (clearance 0.1 - 0.15 mm) with 15 passes of the plunger at 2,000 r.p.m. on a vortex Waring blender (M.S.E. Ltd.). Each homogenate was spun at 3,000 r.p.m. in a

bench centrifuge (M.S.E. Ltd.) for 10 mimutes. The supernatant was decanted and stored at -20°C for ca. 4 days. Previous work has shown that the activity of insect digestive enzymes is stable over much longer periods of storage (Brookes, 1961; Engelmann, 1968). Prior to use they were thawed at room temperature, appropriately diluted with distilled water and kept on ice. During the above procedure all materials were kept on ice when not being homogenised or centrifuged.

Assay of α-glucosidase activity

2 ml aliquots of McIlvaine's buffer (citric acid 0.1 M, Na_2HPO_4 0.2 M) (Dawson *et al.*, 1969) at pH 6.4 (pH of the midgut of adult *Tenebrio molitor* (Srivastava and Srivastava, 1961)) were equilibrated at 30°C with 0.2 ml of homogenate. The reaction was started by the addition of 0.2 ml of 30 mM p-nitrophenol-α-D-glucoside and experiments were incubated routinely for 15 minutes.

The enzyme reactions were terminated by the addition of 3.0 ml of 0.1 M ammonium hydroxide, this also brought the solutions to a pH of 8.5. The latter was necessary to produce the full development of the yellow colour of the nitrophenol, liberated by the action of the enzyme on the substrate (Aizawa, 1939). Substrate blanks were performed by adding the substrate after the ammonium hydroxide. Homogenate blanks were found to be unnecessary as particulate matter in the preparation made

no significant contribution to the absorbance. The yellow colour produced was found to be stable at room temperature over the period of 30 minutes it took to read the absorbance on a CE 505 double beam spectrophotometer (Cecil Instruments Ltd.) at 405 nm. The p-nitrophenol released was determined by reference to a calibration graph prepared by assay of standard p-nitrophenol solutions serially diluted from a stock solution containing 10 μmoles/ml and made up to 5.4 ml with 3 ml of 0.1 M ammonium hydroxide (see Fig. 1).

Statistical techniques

Statistical comparisons of data were performed using the Student's t-test as described by Snedecor and Cochran (1967). Where appropriate reference was made to the statistical tables of Fisher and Yates (1963). Values of probability < 0.05 were taken as being significant.

Experimental animals

Mixtures of male and female insects were used as no significant differences were found between enzyme titres of the two sexes.

Plate 1. Dissection of the alimentary canal of a fed adult male *Tenebrio molitor*

Legend: mp, mouthparts, an, antennae; fg, foregut; mg midgut; hg, hindgut; sd,sperm duct; las, last abdominal segment; mt,Malpighian tubules.

Large arrows define the extent of the midgut.

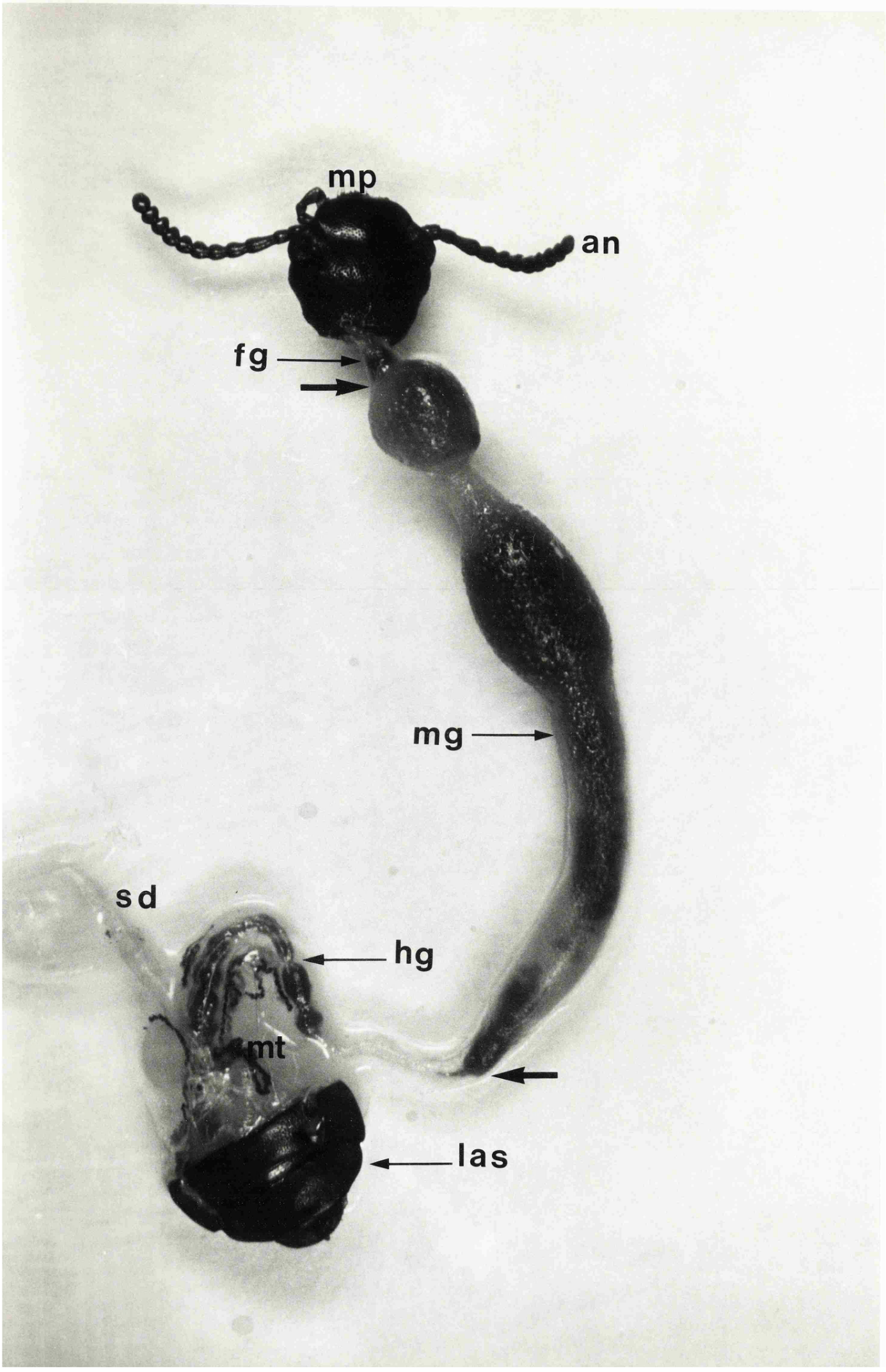

mp
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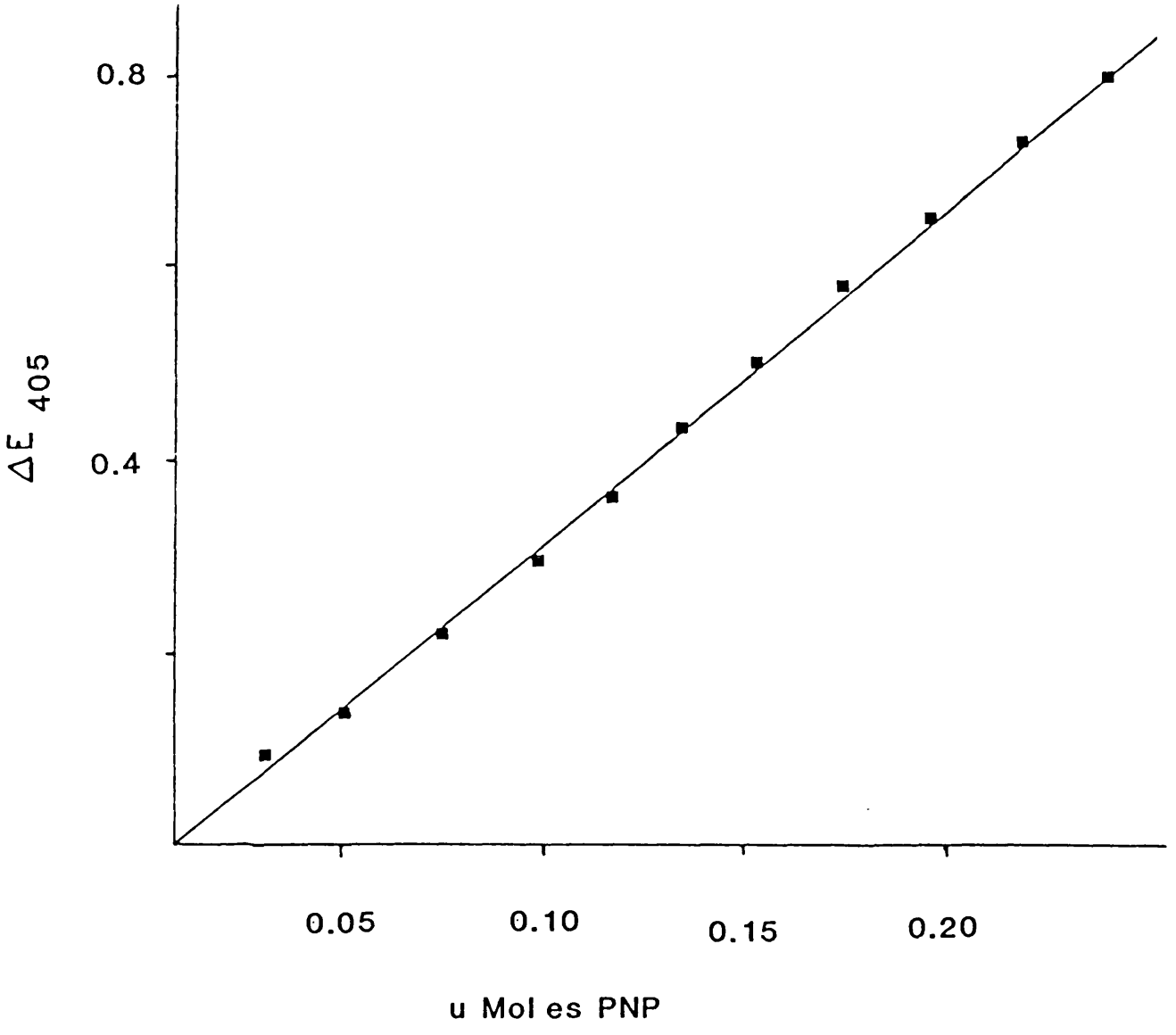
Fig. 1. Standard calibration graph for the determination of p-nitrophenol released from artificial substrate by α-glucosidase.

Method. A standard p-nitrophenol solution was serially diluted from a stock solution, containing 10 μmoles/ml with 2 ml of McIlvaine's buffer (see text) and brought to pH 8.2 with 3 ml of 0.1 M NH_4OH.

Legend: Ordinate: absorbance at 405 nm

Abscissa: μmoles p-nitrophenol


0.8
0.4
ΔE 405
0.05
0.10
0.15
0.20
u Moles PNP

Results

1. Distribution of α-glucosidase activity in the alimentary canal

Most of the enzyme activity was present in the midgut (82%), with only 14% of the total activity in the foregut and 4% in the hindgut (Fig. 2). The anterior midgut contained the most tissue enzyme (Fig. 3).

2. Development of midgut α-glucosidase activity in young beetles

Newly moulted adults had little total α glucosidase activity, but in the first few days of adult life there was a dramatic increase, whether insects were fed (Fig. 4) or starved (Fig. 5). Paradoxically starved animals appeared to have more enzyme activity than fed animals. However, individuals making up the two treatments were taken from different batches of insects which may have had inherently different levels of enzyme activity. Both starved and fed insects exhibited a six-fold increase in enzyme activity from days 1 - 4 of adult life, and it would seem reasonable to conclude that the increase was spontaneous. In fed insects the increase in activity continued up to a peak on the 7th day whereas in starved insects the zenith occurred on the 4th day.

3. The effect of neck-ligation on the development of α-glucosidase activity in young beetles

Abboud (1981) found that neither removal of the head 1 day after nor 2 h prior to the last ecdysis affected the spontaneous increase in enzyme activity which occurs in the first four days of adult life (see Section 2 above). In contrast, ligation of the neck 1-3 h after the pupal ecdysis prevented the increase (Fig. 6).

4. Effect of the timing of neck-ligation on α glucosidase activity in 4 day old adult beetles

Abboud (1981) established that cautery of the pars intercerebralis (pi) 24 h before the adult ecdysis prevented the normal increase in midgut enzyme activity in the first few days of adult life. However, as the timing of the operation was brought progressively closer to ecdysis, the operation had less effect on enzyme activity present in the midgut on the 4th day of adult life. The RT_{50}* for the operation was -3.8 h (see Table 1).

In the present work, application of neck ligatures 24 h before adult ecdysis also had a depressive effect on adult enzyme titre similar to that of ablation of the pi (Fig. 7A). However , the

*The time of the operation which resulted in a 50% reduction in enzyme activity.

critical period for the head in adult midgut enzyme activity (RT_{50}) was -11 h.* There is no reason to believe that cauterisation of the pi is substantially different from the removal of the head in its effects on enzyme activity (Abboud, 1981). Therefore the difference in RT_{50} between Abboud (1981) and this work may be attributed to the larger number of observations made in the latter case, which as a consequence provides the definitive value.

5. Correlation between the effects of neck-ligation on post-ecdysial enzyme activity and tanning

The timing of the pharate adult critical period for the head in midgut α-glucosidase activity is consistent with the hypothesis that one of the developmental hormones is responsible for the spontaneous increase in enzyme activity in the first few days of adult life (see also introduction, "Factor 1").

Of the established developmental hormones, bursicon is released post-ecdysially in most insects including *Tenebrio* which would seem to exclude its candidature (Chapter 2). However, when the tanning scores of the neck-ligated insects used in the previous experiment (Section 4) were determined prior to dissection, a critical period for the head in tanning was found (RT_{50} = -11 h) (see Fig. 7B) which was similar to that in midgut α glucosidase activity (RT_{50} = -11 h; see Fig. 7A). From which it may be construed that the two

* taking into account the delaying effect of neck-ligation on ecdysis (see page 27)

phenomena could have a common mechanism of control.

It was suggested in Chapter 2 that the critical period for the head in tanning represents not the release of bursicon itself, but another previous endocrine event which is required for the acquisition of competence to respond to bursicon, n.b. release of bursicon is not affected by neck-ligation (see Chapter 2). Consistent with this hypothesis, Delachambre (1971) found that transfusing neck-ligated pharate adults with normal "active" blood within 2 days of ecdysis promoted tanning. Unfortunately experiments described in Chapter 2 failed to confirm the existence of Delachambre's Factor (DF),with respect to tanning, and it was decided to see if pharate adult blood and nerve cord extracts could enhance α-glucosidase activity in neck-ligated insects.

6. Effect of blood transfusions on α-glucosidase activity in the midguts of neck-ligated 4-day old beetles; neck-ligation performed 24 h before the adult ecdysis

Injection of 4 µl of -3 h normal blood into -24 h neck-ligated *Tenebrio*, 3 h before ecdysis, caused a significant elevation of α-glucosidase with respect to saline injected controls in the midgut of these animals on the 4th day of adult life (Table 2). The age of donor and recipient may be critical because none of the other samples tested (see Table 2) caused a significant increase in enzyme activity.

7. Effect of blood transfusions and nerve cord extracts on α-glucosidase activity in the midguts of neck-ligated 4-day old beetles: neck-ligation performed 1 - 3 h after pupal ecdysis.

1 - 3 h neck-ligated insects injected with a -24 h nerve cord extract 3 h before they ecdysed had a significantly greater midgut α-glucosidase activity 4 days after ecdysis than saline injected controls (P = 0.02 - 0.01) (Table 3). As in the previous experiment age of donor and recipient may be important because - 12 to -6 h and -24 h extracts injected into 1 - 3 h neck-ligated insects, 18 h before ecdysis, did not significantly affect enzyme titre. Enzyme activity of saline injected extracts was not significantly different from that of controls. -12 h blood did not elevate the enzyme titre of 1 - 3 h neck-ligated pupae when they were transfused 12 h before adult ecdysis.

8. Effect of blood transfusions on the development of α-glucosidase activity in the midguts of neck-ligated young adults: neck-ligation performed 1 - 3 h after the pupal ecdysis

Only blood taken from -3 h normal insects injected into -3 h donors (neck-ligated at -24 h) caused a significant elevation of midgut enzyme titres by the 4th day of adult life (Table 2). However, it seems from Fig. 8 that blood from younger pharate adults can cause a temporary increase in enzyme titre; 1 - 3 h neck-ligated insects injected with -12 h normal blood, 12 h before

ecdysis, had a significantly larger midgut α-glucosidase titre than saline injected insects 2 days after ecdysis but not at any other time ($P = 0.05 - 0.02$).

Fig. 2. Distribution of total α-glucosidase activity in the alimentary canal of mature fed beetles.

Legend: Ordinate: μmoles p-nitrophenol/gut region/minute

Abscissa: F , foregut; M, midgut; H, hindgut

Figures in parentheses denote number of animals

Vertical lines denote 1 S.E. of the mean

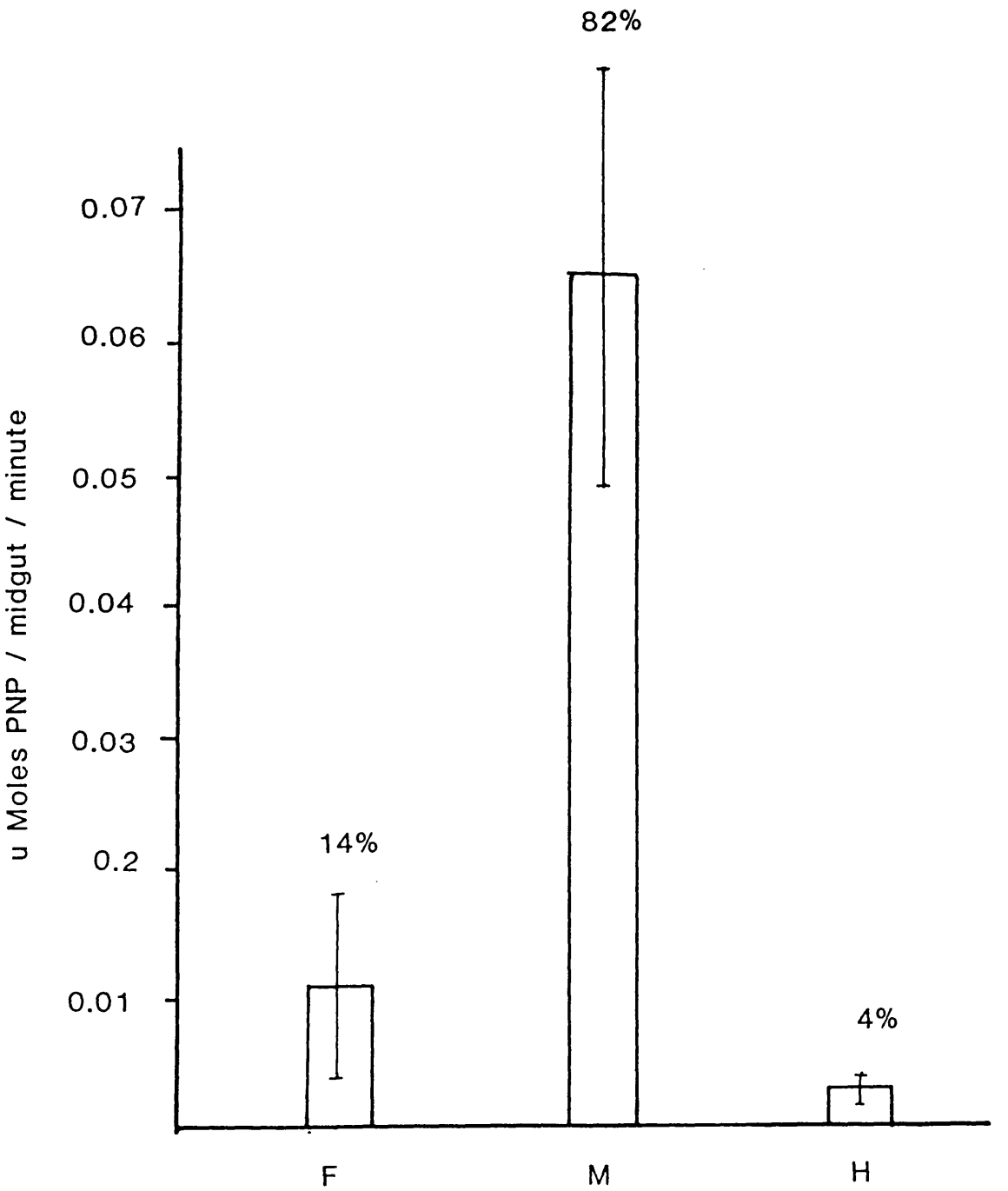

82%
0.07
0.06
0.05
0.04
0.03
0.2
0.01
u Moles PNP / midgut / minute
14%
4%
F
M
H

Fig. 3. Distribution of α-glucosidase activity in the midgut tissue of mature adult beetles.

Legend: Ordinate: μmoles p-nitrophenol/midgut region/minute

Abscissa: AM tissue of the anterior region of the midgut

MM tissue of the middle region of the midgut

PM tissue of the posterior region of the midgut

Figures in parentheses denote number of animals

Vertical lines denote 1 SE of the mean

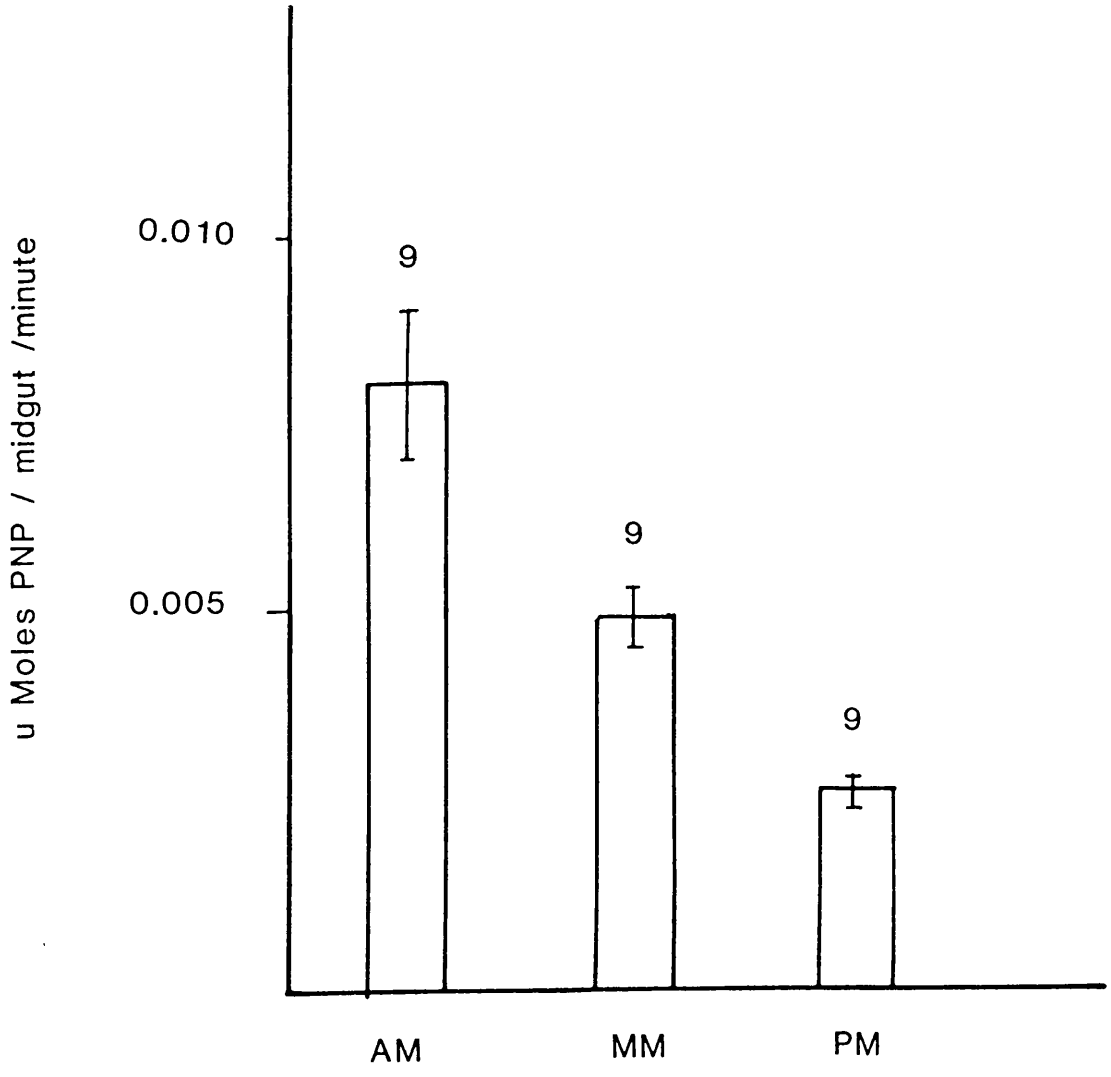

u Moles PNP / midgut /minute
0.010
0.005
9
9
9
AM
MM
PM

Fig. 4. Development of total α-glucosidase activity in young fed beetles.

Legend: Ordinate: μmoles p-nitrophenol/total midgut tissue + contents /minute

Abscissa: days after emergence

Figures in parentheses denote number of animals

Vertical lines denote 1 SE of the mean

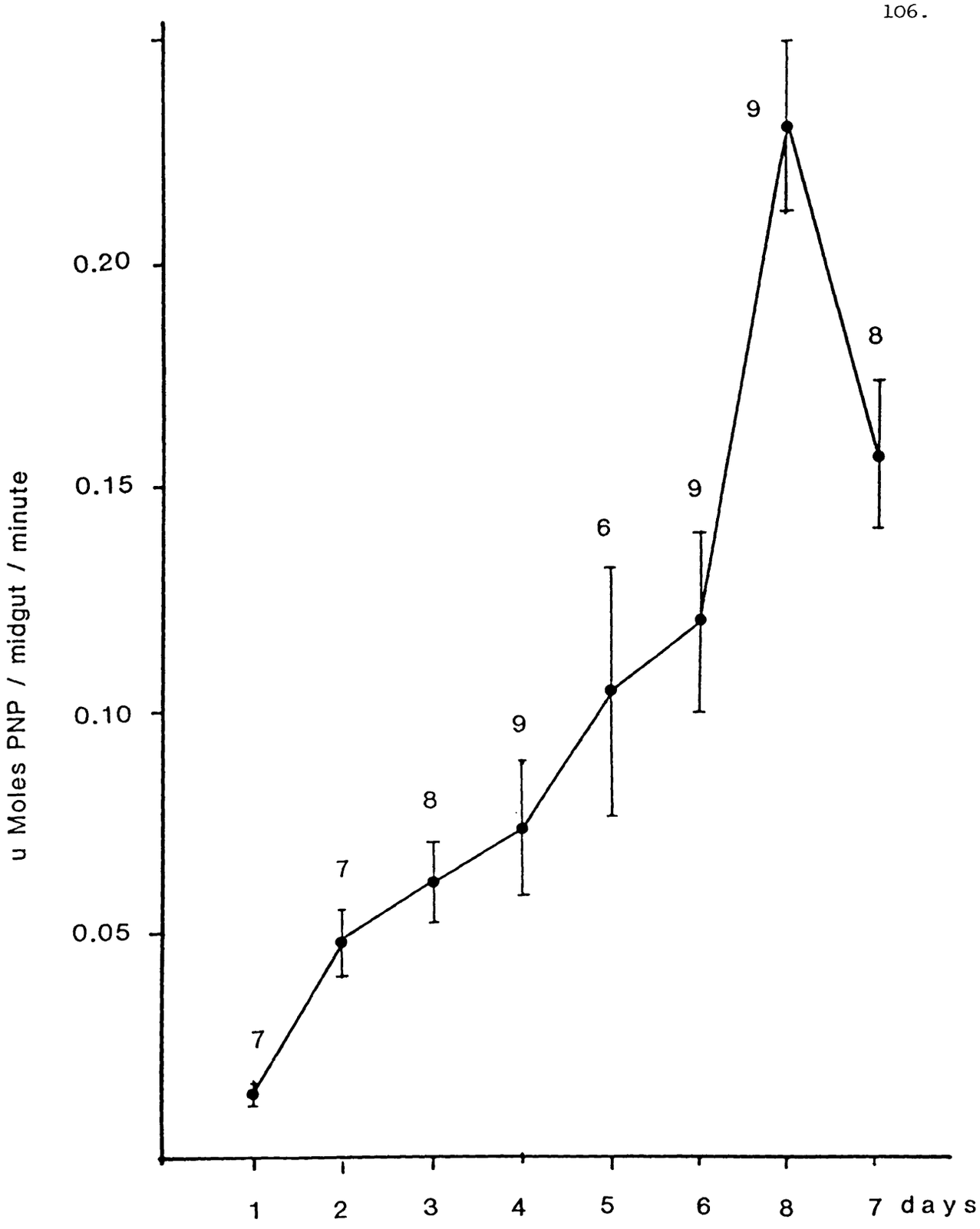

u Moles PNP / midgut / minute
0.20
0.15
0.10
0.05
7
7
8
9
6
9
9
8
1
2
3
4
5
6
8
7 days

Fig. 5. Development of total midgut α-glucosidase in young starved beetles

Legend: Ordinate; μmoles p-nitrophenol/midgut tissue + contents/minute

Abscissa: days after emergence

Figures in parentheses denote number of animals

Vertical lines denote 1 SE of the mean

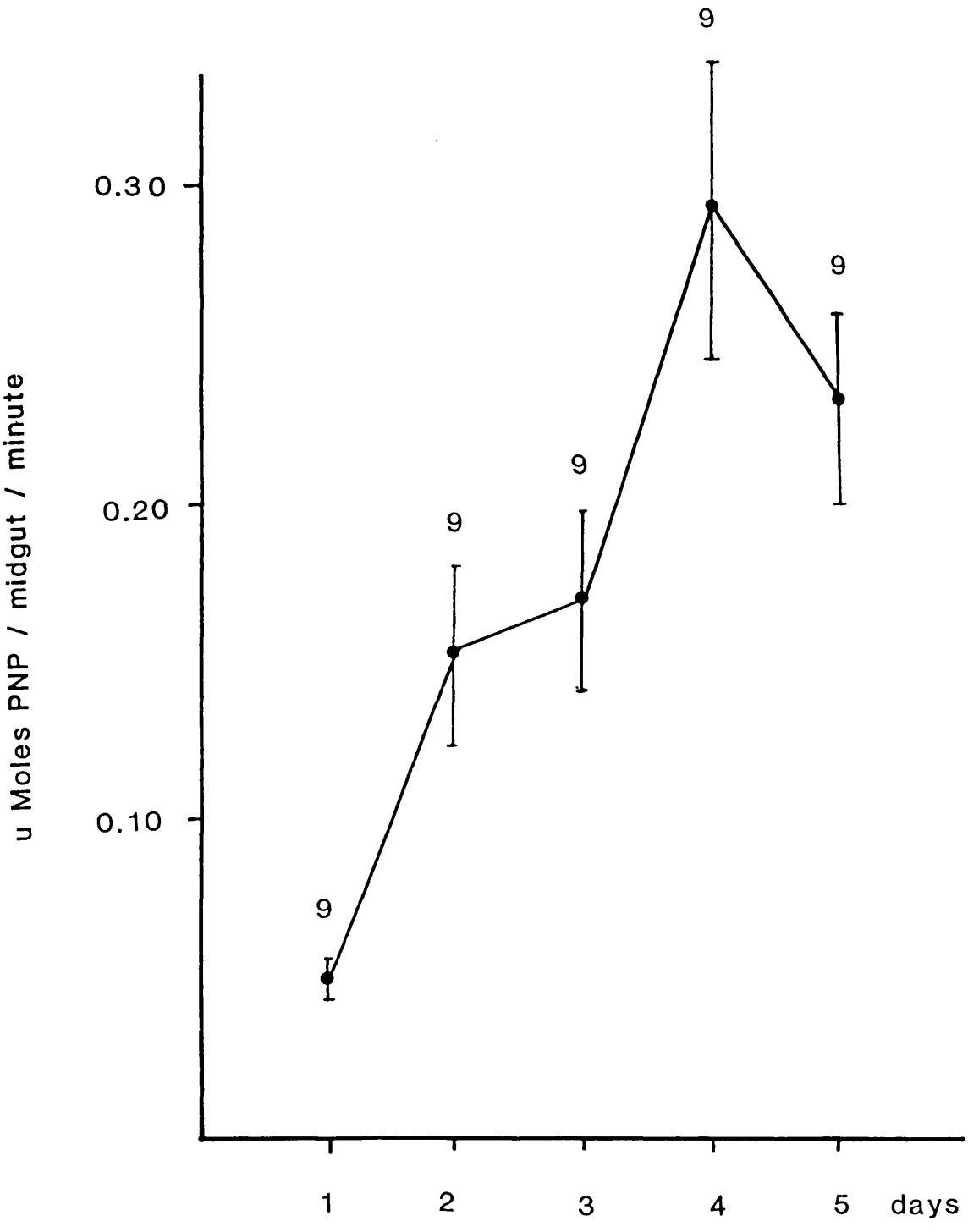

u Moles PNP / midgut / minute
0.30
0.20
0.10
9
9
9
9
9
1
2
3
4
5
days

Fig. 6. Effect of neck-ligation on the development of α-glucosidase in the midgut of young adult beetles

Operation was performed 1 - 3 h after the pupal ecdysis

Legend as for Fig. 4.

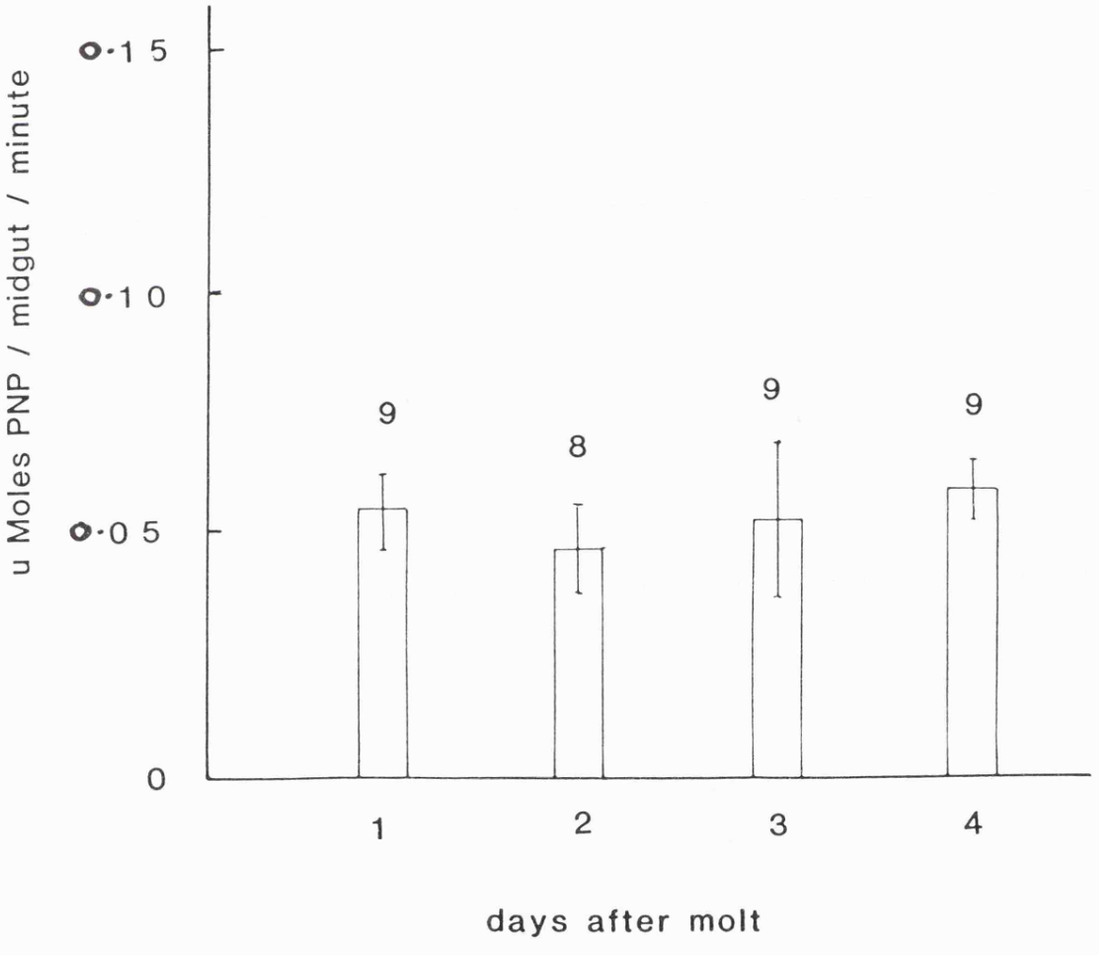

u Moles PNP / midgut / minute
0·15
0·10
0·05
0
9
8
9
9
1
2
3
4
days after molt

Fig. 7. A. The effect of neck-ligation on the α-glucosidase activity in the midgut of 4-day old beetles - operation performed at different times before ecdysis.

Legend: Abscissa: time at which ligature was applied in hours before ecdysis

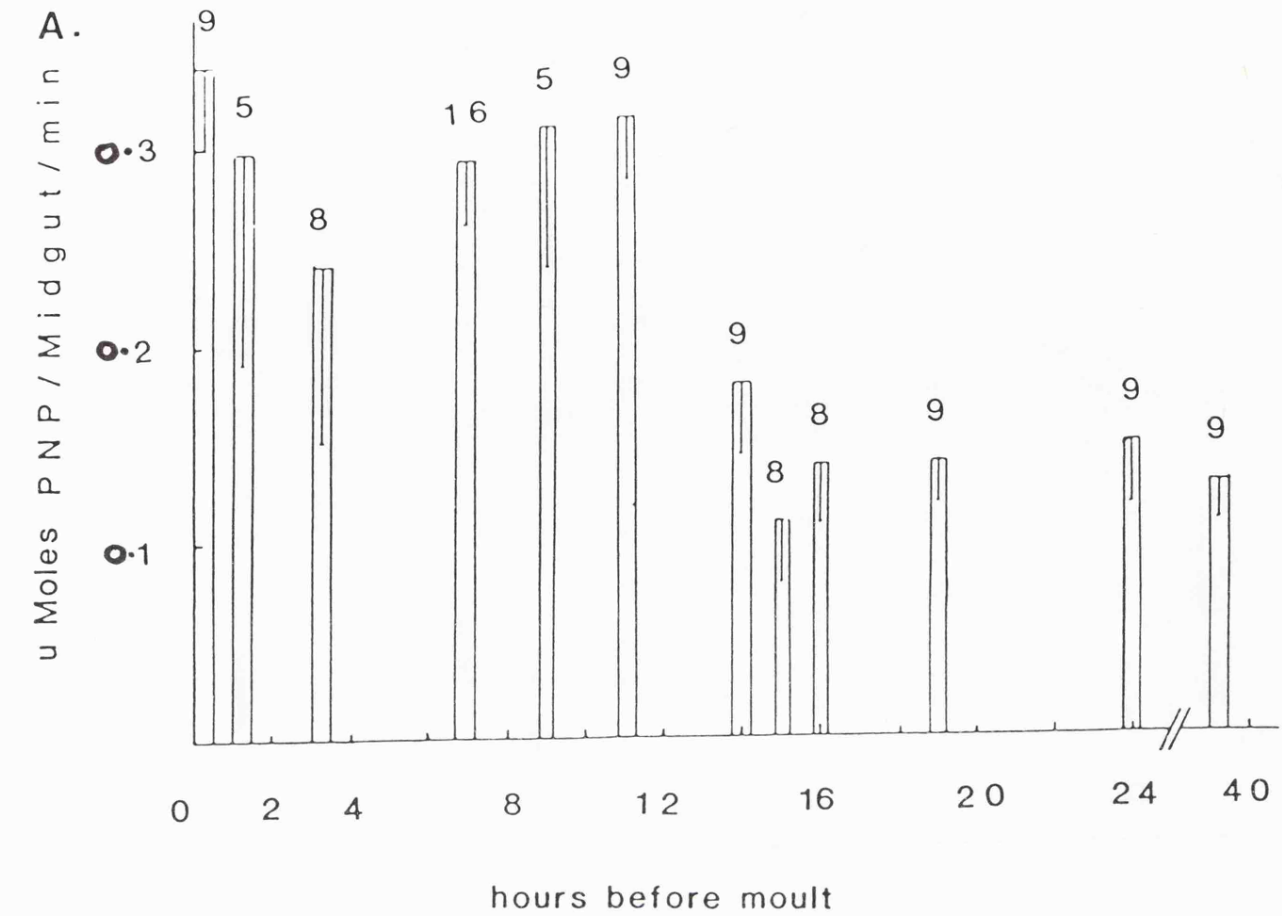
Ordinate: μmoles p-nitrophenol released/ midgut (tissue and contents)/min.

Figures in parentheses denote number of animals

Vertical lines denote 1 SE of the mean

B. The effect of neck-ligation on tanning

Legend as for Fig. 5; Chapter 2.


A.
u Moles P N P / M i d g u t / m i n
0·3
0·2
0·1
9
5
8
16
5
9
9
8
8
9
9
9
0 2 4 8 12 16 20 24 40
hours before moult

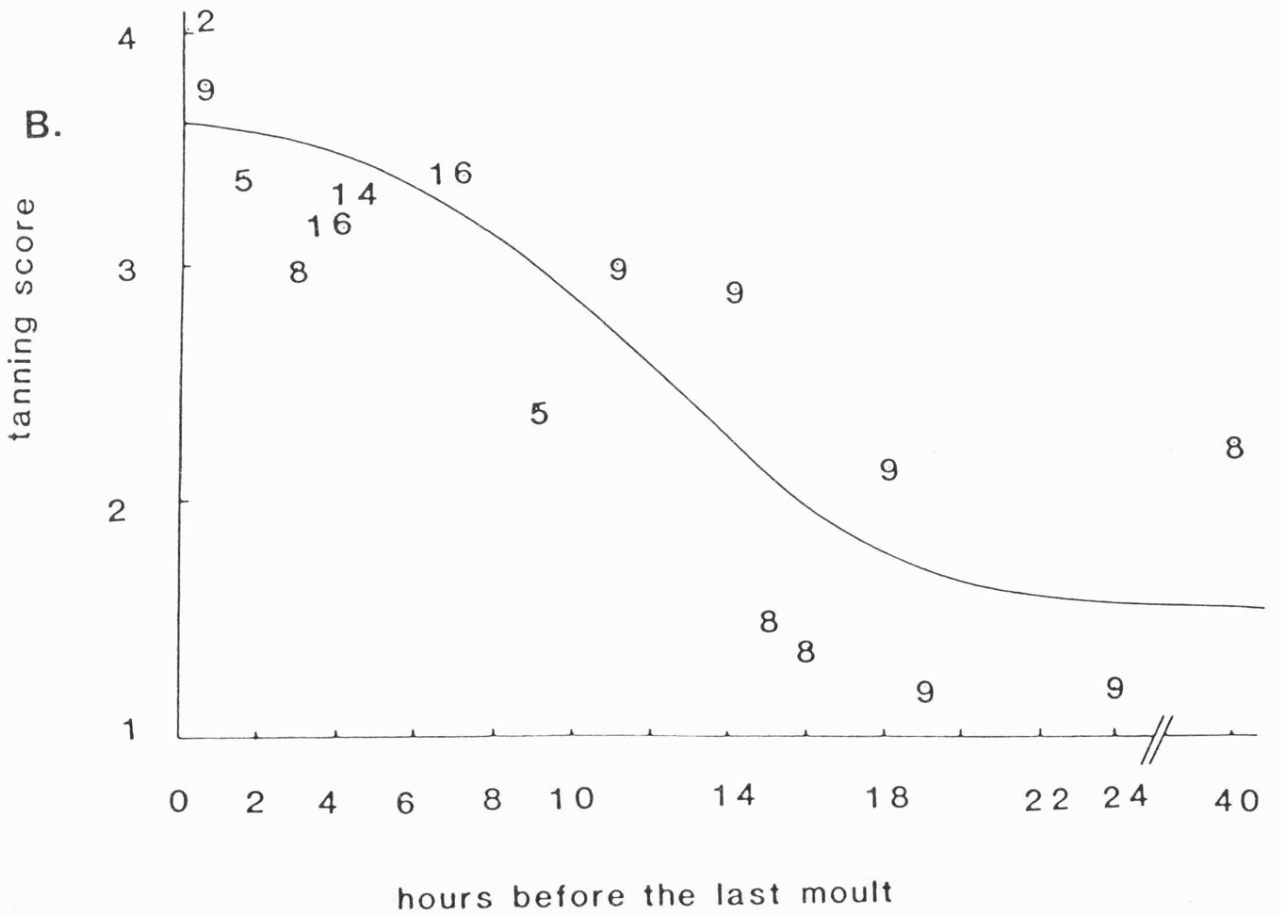

B.
tanning score
4
3
2
1
2
9
5
14
16
16
8
9
9
5
9
8
8
8
9
9
0 2 4 6 8 10 14 18 22 24 40
hours before the last moult

Table 2. Effect of blood transfusions on α-glucosidase activity in the midguts of neck-ligated 4-day old beetles.

Neck-ligation performed 24 h before the adult ecdysis.

Treatment	Age of recipient (h before adult ecdysis)	Age of donor (h before adult ecdysis	Mean enzyme activity (μmoles p-nitrophenol released midgut^{-1} min^{-1})	SE	N
1. blood	-3	-1 to -3	0.09	0.01	7
2. blood	-3	-3	0.19	0.01	7
3. blood	-12 to -6*	-12 to -6	0.145	0.04	7
4. blood	-12	-12	0.1	0.01	7
5. saline	-3	-	0.09	0.01	7

Statistical analysis:

	t	P	
1. blood v saline	1.34	0.3 - 0.2	not significant
2. blood v saline	7.07	< 0.001	highly significant
3. blood v saline	1.335	0.3 - 0.2	not significant
4. blood v saline	0.707	0.5 - 0.4	not significant

* A mixture of 2 developmental stages viz. 6 and 12 "late" (see Table 1, general materials and methods)

Table 3 continued

Statistical analysis:

	t	P	
1. blood v 2.saline	0.128	0.9 - 0.8	not significant
3. nerve cord v 4.saline	2.634	0.02 - 0.01	significant
5. nerve cord v 7. saline	0.756	0.5 - 0.4	not significant
6. nerve cord v 7. saline	0.212	0.9 - 0.8	not significant
5. nerve cord v 6.nerve cord	0.588	0.6 - 0.5	not significant
7. saline v 8. control	1.293	0.3 - 0.2	not significant

Table 3. Effect of blood transfusions and nerve cord extracts on α-glucosidase activity in the midguts of neck-ligated 4-day old beetles: neck ligation performed 1 - 3 h after pupal ecdysis

Treatment	Age of recipient (h before adult ecdysis)	Age of donor (h before adult ecdysis)	Mean enzyme activity (μmoles p-nitrophenol released midgut^{-1} min^{-1})	SE	N
1. blood	-12	-12	0.092	0.012	7
2. saline	-12	-	0.09	0.01	8
3. nerve cord	-3	-24	0.198	0.04	8
4. saline	-3	-	0.09	0.01	7
5. nerve cord	-18	-12 to -6 *	0.112	0.028	7
6. nerve cord	-18	-24	0.092	0.02	6
7. saline	-18	-	0.086	0.02	6
8. control	-	-	0.059	0.006	9

* See Table 2.

Fig. 8. Effect of blood transfusions on the development of α-glucosidase activity in the midguts of neck-ligated young adults; neck-ligation performed 1 - 3 h after the pupal ecdysis. Blood taken from -12 h normal insects and injected into experimental insects at -12 h.

Legend: Abscissa: days after ecdysis

Ordinate: μmoles p-nitrophenol released/ midgut/min

Vertical bars denote standard error of the mean

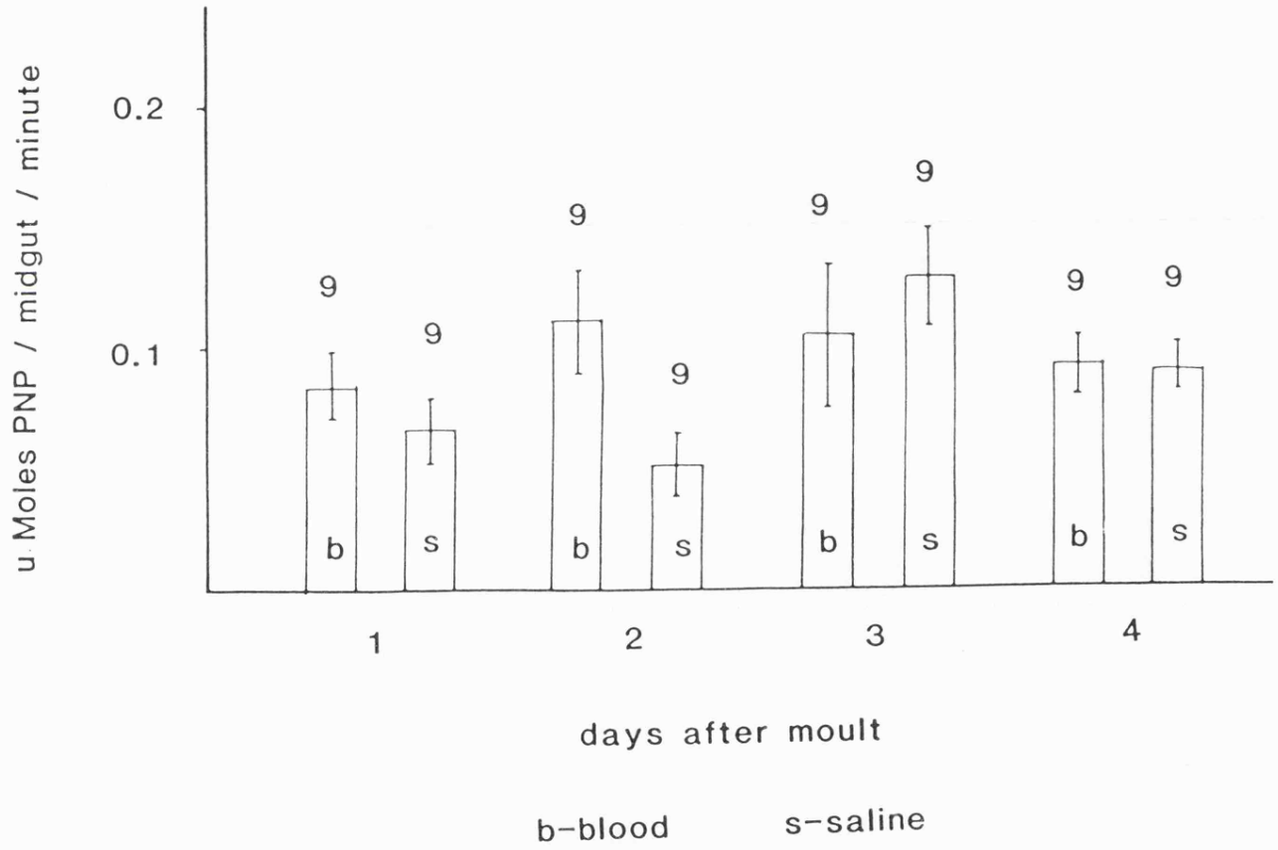
Figures denote number of determinations

b = injected blood

s = injected saline

Statistical comparisons of data		t	P
Day 1	blood v saline	0.746	0.5 - 0.4 ns
Day 2	blood v saline	2.46	0.05 - 0.02 s
Day 3	blood v saline	0.667	0.6 - 0.5 ns
Day 4	blood v saline	0.177	0.9 - 0.8 ns

s = significant; ns = not significant


u Moles PNP / midgut / minute
0.2
0.1
9
9
9
9
9
9
9
9
b
s
b
s
b
s
b
s
1
2
3
4
days after moult
b-blood
s-saline

Discussion

The crop is the main site of digestion in the Orthoptera (Evans and Payne, 1964; Anstee and Charnley, 1977), digestive enzymes from the midgut are passed forward and are joined by secretions from the salivary gland; the same is true of Carabid beetles (Wigglesworth, 1972). However, in *Tenebrio molitor* the foregut is a simple tube and contains little α-glucosidase activity. Consequently the midgut with over 80 % of the enzyme activity must be the most important site of digestion in the adult beetles. The anterior midgut tissue may be the main site of synthesis of α-glucosidase, as there was more activity of this enzyme species here than in the tissue of either of the other two midgut regions. Dadd (1954) also found amylase activity concentrated anteriorly in the midgut tissue of *Tenebrio* larvae and adults, while protease showed a posterior concentration.

The results presented here show that newly moulted adult beetles have only a small amount of total midgut enzyme activity, but in the first few days of adult life there is a dramatic increase, whether the insects are fed or starved. Although both starved and fed treatments exhibited a 6-fold increase in enzyme activity during days 1 - 4 of adult life, the absolute enzyme levels were very different. Paradoxically the starved had a greater amount of enzyme activity than the fed. Since different batches of insects were used for the two treatments the difference in enzyme activity may have been in-

herent, rather than a reflection of the treatments. Certainly Dadd (1956) reported a spontaneous increase in protease activity in young adult *Tenebrio*, viz. the increase occurred whether the insects were fed or starved. Similar developments in digestive enzyme activity in newly ecdysed adult insects have been found in *Glossina morsitans* (Langley, 1967) and *Locusta migratoria* (Khan, 1964).

It has been shown both in the present work and by Abboud (1981) that there is a critical period in the 24 h prior to the adult ecdysis during which the head must remain intact for the normal post-ecdysial increase in α-glucosidase activity to occur. The detailed experiments described here established an RT_{50} for the operation of -11 h.

Dadd (1961) showed that protease activity also failed to develop in the midguts of adults which had been decapitated 1 day before emergence. A few experiments suggested that injection of the blood of fed or normal newly emerged adults (but not starved insects) elevated protease in starved adults. Therefore he concluded that a humoral factor was involved. However, Abboud's (1981) ligation experiments are consistent with the existence of 3 factors (1 pre-ecdysial and 2 post-ecdysial) acting sequentially to control the development of α-glucosidase in young adults. In the light of this Dadd's (1961) attempts to relate the effects of pre-ecdysial neck-ligation and post-ecdysial blood transfusion experiments on enzyme titres are now seen to be ill-founded.

The present work adds credence to the view that a hormone (Abboud's (1981) - 'Factor 1') is released prior to ecdysis, that influences the development of the midgut digestive enzyme activity after emergence. Transfusion of "active" blood promoted the development of enzyme activity in neck-ligated insects. However, the age of donor and recipient appear to be critical, suggesting that appearance of the factor in the blood and responsiveness of the insect are reasonably brief events.

"Factor 1" also appeared to be present in the nerve cord. However, elevated titres of α-glucosidase in (1-3 h) neck-ligated beetles injected with -24 h nerve cord extract at -3 h may be contrasted with the enzyme activity in those individuals treated with -24 h extract at -18 h, which was not significantly different from the controls (Table 3); clear evidence that the insect is responsible for only a brief period.

If "factor 1" is a hormone, what is its nature? Dadd (1961) concluded that the "humoral factor" was either a special hormone or one of the hormones which "primarily function in the regulation of growth, metamorphosis and adult maturation. The latter possibility seems more likely in view of the spontaneous development of midgut enzyme activity, while adult characters reach their full expression during the few days following emergence."

However, JH and PTTH may be discounted because they are not present at the appropriate time (see for example the review by

Richards (1981)) and ecdysone may be discounted because blood titres are unaffected by abdominal ligation (Delbecque *et al.*, 1978). Although it was shown in Chapter 2 that bursicon is released post-ecdysially, thus ruling it out as being "factor 1", a relationship between tanning and post-ecdysial midgut enzyme activity is indicated by the similarity of the critical periods for the head in these two events. It was suggested in Chapter 2 that the critical period for the head in tanning represented the release of another hormone that confered competence to respond to bursicon. This was thought to correspond to Delachambre's (1971) factor (DF), and the hypothesis was made that DF is an eclosion hormone (EH). Some circumstantial evidence was obtained which was consistent with an EH though blood samples and nerve cord extracts failed to promote tanning or accelerate ecdysis in neck-ligated insects. It is tempting to ascribe a further function to DF namely the control of the spontaneous increase in enzyme activity in early adult life viz. "factor 1" and DF are synonymous. Confirmation of this relationship will depend on the extraction and purification of the factor(s) responsible.

CHAPTER 4

THE ULTRASTRUCTURE OF THE ENTEROCYTES OF THE MIDGUT OF FED AND STARVED *TENEBRIO MOLITOR*

Introduction

An extensive literature exists on the organisation of the midgut in a wide variety of insects (reviewed by Wigglesworth, 1972; Smith, 1968). The midgut consists primarily of columnar cells (enterocytes) which bear microvilli along their apical surface bordering the lumen. This epithelium rests on a basement membrane and is invested with circular and longitudinal muscle fibres. In *Periplaneta americana* the basal region of the cells is deeply dissected by irregular infoldings of the cell membrane, and many mitochondria lie in the resulting narrow cytoplasmic sheets. The extracellular spaces formed by these infoldings, in *Hyalophora cecropia*, may extend two-thirds of the way to the apical border. These invaginations enormously enhance the area of basal plasma membrane possessed by each cell, across which materials may pass from the cytoplasm to the haemolymph and vice versa (Smith, 1968). The microvillar brush-border similarly increases the surface area of the apical membrane.

Along the columnar epithelial cells of the midgut, a smaller number of specialised goblet cells may occur. They are characterised by the invagination of the apical border to form a deep cavity,

confining the nucleus to the basal region. The cavity is lined with microvilli, which, unlike those on the apical surface of adjoining columnar cells, are irregular in thickness and many of them contain mitochondria. These cells occur in Ephemeroptera and Plecoptera, but they are most numerous in larvae of Lepidoptera becoming most numerous towards the hind end of the midgut (Wigglesworth, 1972). At least in *Hyalophora cecropia* goblet cells serve an excretory function, removing excess potassium from the haemolymph, a legacy of the high concentrations of this ion in plants (Anderson and Harvey, 1966; Harvey and Nedergaard, 1964).

Embryonic or replacement cells are also found between active epithelial cells. These may be scattered singly along the gut as in Lepidoptera and Diptera; or they may be found at intervals in small groups or nidi as for example in Orthoptera and Odonta. Nidi may form large crypts projecting through the muscular coat and standing out like villi over the outer surface of the gut, e.g. in many Coleoptera. As the epithelial cells degenerate, the regenerative cells grow to replace them (Wigglesworth, 1972).

In most insects the structure of the midgut is uniform apart from the presence of caeca. In some, however, there is a clear division into functional regions. The most anterior cells of the midgut epithelium of *Calliphora vicina* are involved in peritrophic membrane (pm) formation (Smith, 1968). They

are tall and each fans out from a narrow basal region to a broader apical surface. The nuclei lie in the expanded apical half of the cell, and Golgi bodies and rough endoplasmic reticulum are well developed. Vesicles derived from the Golgi bodies congregate along the apical surface of the cell, and appear to discharge their contents into the extracellular space between the microvilli. An outer ring of columnar cells is similarly constructed, but appears to manufacture a more homogeneous, opaque secretion, perhaps a second constituent of the membrane that forms in the extracellular cleft between the two epithelial rings. As the first component of the peritrophic membrane passes by the surface of the oesophageal invagination, it is joined by a second sheet that comes away from the foregut cells. A third component of the pm is a thin membrane formed by the normal unspecialised epithelial cells. It should be noted that in contrast to Smith's (1968) detailed description of the origin of the type II pm in *C. vicina*, there is little known about the cellular origin of the more numerous type I pm (see Discussion).

The larvae of *Lucilia cuprina* have a long coiled gut consisting of three segments: the anterior and posterior, which are similar histologically and consist of vacuolated cells, secrete digestive enzymes; the short middle zone is strongly acid but secretes no enzymes. The second zone of the middle of the midgut of larval *Lucilia cuprina* is a mosaic of two types of cell (Waterhouse and Stay, 1955); a) cells with an unusual striated border, their cytoplasm full of lipid spheres and glycogen, rich

n acid phosphatase (lipophilic cells); b) cells with an inconspicuous striated border, rich

in esterases, which specifically accumulate iron and copper (cuprophilic cells). In all, five zones in the middle of the midgut of the *Lucilia* larva have been distinguished histochemically (Poulson and Waterhouse, 1960).

A clear division of labour is seen in the midguts of adult *Glossina morsitans* (Wigglesworth, 1929) and Capsid bugs (Goodchild, 1953). In both of these guts there is a sac-like region in which the ingested food is concentrated, a tubular region which is the chief site of digestion, and a third region specialised for absorption. A characteristic arrangement of the midgut exists in Homoptera, associated with a diet of dilute plant juices (Cheung and Marshall, 1973a). The terminal region of the midgut comes into intimate relation with the first region. This enables excess fluid in the food to pass directly from the first part of the midgut to the last without diluting the haemolymph or the sites of absorption. This morphological differentiation is matched by a considerable ultrastructural and cytochemical differentiation in the adult cicadoid and larval cercopoid midgut (Cheung and Marshall, 1973b). It is suggested that this may be related to functions such as ion absorption and secretion, mineral storage excretion, nutrient absorption and intermediary metabolism.

Significant variation in cell structure may occur when the midgut appears morphologically quite uniform e.g. larval *Manduca sexta* (Cioffi, 1979). The midgut of *Manduca* can be

divided into anterior, middle and posterior regions on the basis of the pattern of folding of the epithelial sheet, and variations in the structure of goblet and columnar cells which occur along its length. The microvilli of anterior columnar cells form a dense, interconnecting network from which vesicles break off. This organisation becomes less obvious through the middle region, until by the posterior region each microvillus is unconnected to adjacent microvilli along its entire length and vesicles are no longer produced. Two distinct types of goblet cell are found. In the anterior and middle regions the goblet cells have a large basally located cavity,but in the posterior region the cavity occupies only the apical half of the cell. It remains to be seen how such variation in fine structure is related to function.

There is no phagocytosis of food particles by the midgut cells of insects (Wigglesworth, 1972). Although pinocytosis has been demonstrated in larvae of *Ephestia kuhniella* (Smith *et al.*, 1969), it is thought that in the main nutrients are absorbed as small molecules directly across the apical plasma membrane (Smith, 1968). In most insects there is no doubt that secretion of digestive enzymes and absorption of nutrients are carried out by the same cells (Wigglesworth, 1972). However, in others there is a division of labour nb. *Glossina morsitans* - see above. In some insects cells are thought to go through alternative phases of secretion and absorption e.g. *Periplaneta americana* (Gresson, 1934) but the evidence is

not conclusive (Wigglesworth, 1972). More recently Zimmerman *et al.* (1969) reported having distinguished three functional stages in the midgut cells of *Calliphora vicina*, viz. the secretion of digestive enzymes, the production of a mucoproteid substance, and the release of this substance by delamination (pm). However, De Priester (1971) was unable to confirm their observation that these processes occurred at different times.

The process of enzyme secretion in the insect gut has often been described on the basis of cytological observations made by light microscopy (reviewed by Wigglesworth, 1972). Many investigators noted a process of vacuolation in the apical halves of midgut cells. The subsequent release of the vacuoles as cytoplasmic globules or extrusions from the epithelium into the lumen was assumed to be the visible evidence of the release of enzyme. Secretion has been described as merocrine when the nucleus is retained and only cytoplasmic globules are extruded, and holocrine when the entire cell breaks down, liberating its contents, including the nucleus, into the lumen. Critics of this theory have proposed that these phenomena are either fixation artefacts or result from the disintegration of cells, and do not represent a secretory process (Wigglesworth, 1972). Day and Powning (1949) reported that the occurrence of cytoplasmic extrusions from midgut cells of *Blatella germanica* was not correlated with an increase in enzyme activity in the gut lumen. Indeed the greatest concentration of enzyme occurred when the midgut epithelium was cytologically uniform. Khan and Ford (1962) showed that

cytological changes involving vacuolation occurred as a response to starvation in the gut cells of *Dysdercus fasciatus*.

The electron microscope has proved a useful tool in uncovering the mechanism of protein secretion in the mammalian exocrine pancreas (Caro and Palade, 1964; Jamieson and Palade, 1967a,b). A well developed endoplasmic reticulum synthesizes the protein, partitions it from the rest of the cellular cytoplasm, and transports it to a prominent Golgi apparatus. Here the protein is packaged into membrane bound vesicles (mvb) which carry the product to the secretory border of the cell for export. In the ultrastructural studies on the midgut cells of insects carried out so far, a well developed rough endoplasmic reticulum and Golgi apparatus have often been described but the presence of large numbers of mbv, or indeed the vacuolation process described by light microscopists have not (Smith, 1968). It must be concluded that secretion can take place in the midgut without the cells showing any alteration (Wigglesworth, 1972).

In contrast to the general failure to find ultrastructural evidence for enzyme secretion is a recent study by Lehane (1976) on the haematophagous fly *Stomoxys calcitrans*. He has shown that the opaque zone midgut cells are responsible for the production of proteolytic digestive enzymes and that these are secreted into the gut lumen via mbv. The secretory cycle can be summarised as follows: initially the rough endoplasmic reticulum is stacked and the apices of the cells are packed

with mbv. Following the ingestion of a blood meal release occurs first by cytoplasmic extrusions containing high densities of mbv, then by microvesiculation of the microvilli, this is accompanied by a progressive distribution of rough endoplasmic reticulum and lightening of the cellular cytoplasm. Glycogen appears in the cells at this stage and is gradually lost as the rough endoplasmic reticulum becomes stacked once more and the numbers of mbv build up again. The cycle which occurs regularly and synchronously in the cells of the zone repeats itself many times up to the completion of digestion of the blood meal.

Whorls of rough endoplasmic reticulum have also been reported in *Aedes aegypti* (Bertram and Bird, 1961) and three species of *Anopheles* (Staubli *et al.*, 1966). In mosquitoes the whorls undergo only one cycle of distribution and reconstitution during the digestion of one blood meal. Staubli *et al.* (1966) proposed that the whorls secreted and stored digestive enzymes. However, Lehane (1976) has pointed out that at least in some vertebrate systems increasing organisation of the rough endoplasmic reticulum is correlated with a decrease in secretory activity. He concluded that in *Stomoxys calcitrans* the distribution of the whorls reflected an increase in synthetic activity, while the re-organisation and orientation of the cisternae interrupted synthesis, but was necessary to allow transfer of products to the Golgi bodies. The single cycle of whorling seen in the mosquitoes then correlates

with the absence of production of large numbers of Golgi generated mbv in these insects.

In contrast to *Stomoxys calcitrans* most insects do not store substantial amounts of digestive enzymes (see Introduction to Chapter 3), and it is suggested that enzyme synthesis and release are interdependent processes (House, 1974). This presumably accounts for the absence of Golgi generated mbv. The small amount of enzyme activity that is present in the midgut epithelium must be in the rough endoplasmic reticulum. If mbv are not responsible for the transport of enzymes to and the secretion from the apical membrane, how does it take place? Vesiculation of the microvilli has been described in the midgut cells of *Dacus olea* and *Blabs gibba* (Baccetti, 1962), *Fulgora candelaria* (Marshall and Cheung, 1970), *Calliphora vicina* (De Priester, 1971) and *Sarcophaga bullata* (Nopanitaya and Misch, 1974). These authors also describe vesicles with similar diameters budded from the rough endoplasmic reticulum. Lehane (1976) has suggested that since similar microvesicles in *Stomoxys calcitrans* are certainly secretory it may be that transportation of digestive enzymes in vesicles derived from the rough endoplasmic reticulum and their release from microvilli is a general secretory mechanism in insect cells. This would certainly account for the paucity of Golgi system generated mbv found to date.

Cytoplasmic extrusion, recorded often in light microscopy

studies (see above), has also been found in ultrastructural studies of insect midgut. De Priester (1971) showed that the fine structure of extrusions in the midgut of *Calliphora vicina* depended to a considerable extent on the fixation method used. The vacuolated appearance of the extrusions - often described by light microscopists - was seen only after fixation in hypotonic fixatives, and proved to be caused by dilation of the endoplasmic reticulum and the perinuclear space. In tissue judged on the basis of the appearance of the mitochondria and endoplasmic reticulum to show good preservation of structure, extrusions had the following fine structure: the cytoplasm contained a vesicular form of the endoplasmic reticulum, few mitochondria, and very often a cluster of lysosomes or residual bodies; the microvilli were without exception smaller in size and fewer in number. De Priester concluded that the apical cytoplasmic extrusions reflected a process of degeneration.

Microvesiculation of the microvilli and cytoplasmic extrusion observed in *Calliphora vicina* (De Priester, 1971) are also features of the midgut epithelial cells of larval *Locusta migratoria* refed after forty hours of starvation (Heinrich and Zebe, 1973). Two distinct types of vesicle were seen; large vesicles budded at the top of the microvilli and small vesicles found anywhere in the brush-border forming rows like strings of beads. The view that the former structures are involved with digestive enzyme secretion is supported by the work of Droste and Zebe (1974). They found a dramatic reduction in the proportion of the total

midgut carbohydrase activity in the epithelium following the refeeding of locusts starved for forty-eight hours, 35% down to 2%. This might indicate the release of stored enzyme, however, absolute values were not given. Finally, Heinrich and Zebe (1973) concurred with the view expressed by De Priester (1971) that cytoplasmic extrusions reflected a process of cellular degeneration.

In the main, studies on the relationship between structure and function in the midguts of frequent feeders have had to rely on the feeding of previously starved insects to bring about a sufficiently dramatic increase in digestive enzyme activity, e.g. Heinrich and Zebe (1973). This has led to problems in the interpretation of the fine structure, in particular observed features could be indicative of epithelial renewal and/or enzyme release. It has been reported both in the present study (Chapter 3) and by Dadd (1956) that there is a large spontaneous increase in midgut digestive enzyme activity within the first few days of adult life of *Tenebrio molitor*. Both starved and fed individuals showed the same increase in α-glucosidase activity (Chapter 3) and protease (Dadd, 1956). Therefore, a comparison between the gut ultrastructure of starved and fed insects may provide an insight into the mechanism of digestive enzyme secretion in a frequently feeding insect. Abboud (1981) made provisional observations on the anterior midgut. The present work has extended the work and included middle and posterior regions as well.

Materials and Methods

The animals employed and the treatment of experimental animals were as described in the general materials and methods section (Chapter 1). All reagents used were AnalaR grade or the purest available and supplied by British Drug Houses, or Sigma.

Transmission Electron Microscopy (TEM)

Four-day-old male adult beetles were used in this investigation. They were either fed or starved from emergence for 96 hours then sacrificed and dissected (for the technique see Materials and Methods to Chapter 3). The midgut was fixed for 4 hours in 5% gluteraldehyde in 0.02 M sodium cacodylate at pH 7.2. This was followed by post osmication with 2% OsO_4 in 0.02 M sodium cacodylate (pH 7.2) for a further 4 hours. Fixed tissues were washed with 0.02 M sodium cacodylate for 5 minutes, before being dehydrated by passage through an acetone series; 10 minutes in each of 50%, 70%, 95% and two changes of 10 minutes in absolute acetone. The gut was then transferred to a 50/50 (v/v) mixture of acetone and resin* for two days. Following infiltration in 100% resin for a further two days, the material was embedded in fresh resin and polymerisation was effected at 60°C for 96 hours. Before sections were taken the embedded gut was cut into 3 equal pieces; anterior, middle and posterior.

* Spurr's resin: 10 ml ERL 4206, 6 ml DER 0736, 26 ml NSA and 0.4 ml Si.

Thin sections ("gold coloured") were cut on a Reichert NK ultratome, expanded with ether vapour and mounted on carbon and formvar coated copper grids. Sections were stained with uranyl acetate and lead citrate (Reynolds, 1963) prior to examination in an AEI EM6B electron microscope. Thick sections were placed on glass slides and stained with methylene blue.

The results presented below are representative observations made during the course of an experiment involving three male beetles of each treatment (starved or fed from emergence for 4 days).

Scanning Electron Microscopy (SEM)

The dissected midgut was fixed in 6% glutaraldehyde in 0.05 M sodium cacodylate at pH 7.2 for 3 h. The gut was then passed through a graded series of water/acetone solutions to 100% acetone, dried in a critical point drier (Polaron E3000) and coated with gold using a Polaron Argon Sputter Coater. Examination of the prepared specimens was carried out in a Cambridge S4 Stereoscan SEM at 10 kV.

The results presented below are representative observations made during the course of an experiment involving 3 male beetles (fed from emergence for 4 days).

Results

Histology of the anterior midgut

The midgut consists of an epithelium that contains secretory cells (enterocytes) and large crypts of regenerative cells (nidi) (Plate 1A and B). The epithelium is covered by a coat of connective tissue, inner circular muscles, and outer longitudinal muscles (Plates 1A and 4), through which the nidi project (Plate 1A).

The anterior midguts from insects fed or starved from emergence for 4 days have a similar appearance in low power light micrographs (Plate 1A and B). However, in higher power a number of differences are observed. The apical membrane in the midgut of starved animals is thrown into a series of tight folds with a narrow, but in places deep, channel between adjacent folds (Plate 2B). A brush border is present over the surface of the apical membrane. Many enterocytes are very thin for ca. 90% of their length and arranged radially with respect to the lumen of the midgut. However, proximal to the lumen these cells increase in width and subtend an angle of approximately 90^{o} to the apical membrane (Plate 2B; Fig. 1). This means that where the membrane is indented, the enterocytes bend towards it (Plates 2B and 4; Fig. 1). The thin elongated region of the enterocytes stain deeply with methylene blue. In contrast, the expanded proximal region is only lightly stained. Their pale appearance is intensified by the presence of many small vacuoles (Plates 2B and 4). In the immature secretory cells the nuclei are quite

conspicuous, in particular the chromatin stains deeply with methylene blue. Most of the mature enterocytes have the nucleus in the thin elongated portion of the cell, where its presence is obscured by the intensity of the staining (Plate 2B).

The anterior midgut of fed insects is characterised by the presence of many large cytoplasmic extrusions in the brush border of the apical membrane (Plates 2A and 3; Fig. 1). In many cells the brush border, although still present, is obscured by the extrusions (Plates 2A and 4). Channels between adjacent folds of the apical membrane are less apparent than in the guts of starved insects (compare Plates 2A and 2B). Lamellae of the peritrophic membrane are conspicuous in the midgut of the fed insect, but almost totally absent in the starved (Plates 2A and 2B). Apart from the features mentioned above the histology of the enterocytes of fed and starved insects are similar.

The anterior midguts of both starved and fed insects contain many regenerative nidi (Plate 1A, B). These consist of numerous small cells (Plates 2A,B). There are no apparent differences between nidi from the two treatments.

Histology of the middle and posterior midgut

The appearance of the epithelium in the posterior midgut of both fed and starved insects is substantially the same as that of the anterior midguts of the two treatments (Plate 6A and B).

However, unlike the anterior midgut the apical membrane of the middle midgut (starved or fed) is not thrown into folds (Plates 5A and B). Some of the enterocytes are thin and stained deep blue, most are quite wide and less densely stained; all are arranged radially with respect to the lumen of the midgut. Cytoplasmic extrusions (ce) are present in the middle midgut of fed insects but they are not as numerous as in the anterior and posterior midguts of fed insects. Ce are absent from middle and posterior midguts of starved insects as they are from the anterior midgut of this treatment.

Ultrastructure of the enterocytes

Transmission electron microscopy

Three types of cell are found:

"type 1", few observable structures apart from some membrane bound vesicles (Plate 7).

"type 2", rought endoplasmic reticulum (rer) extensive but not conspicuous, cytoplasm relatively dense (with respect to "type 1" and "type 3" cells), few to many prominent Golgi bodies which may be concentrated in one part of the cell (particularly close to the nucelus) (Plates 8A, 9B, 12A, 18A, 19A and B, 20A, B and C, 21C, 22B, 23B).

"type 3", rer extensive and the cisternae dilated (Plates 7B, 8B, 10, 11B, 12B, 18A and B).

The above descriptions of cell types are not mutually exclusive, Many cells have areas that correspond to several or all three of

the "types" (Plates 17A, 18A). In particular a cell which is substantially "type 2" or "type 3" may have the region close to the apical membrane devoid of organelles apart from mitochondria and membrane bound vesicles (mbv 1 and 2, see later) similar to a "type 1" cell (Plates 9A, 15A).

The cytoplasm of the enterocytes may contain a number of vacuoles, some of which have affinity with lipid droplets (though they may be devoid of osmiophilic material) (Plates 9A, 20A), others seem to be lysosomal (Plate 8A). The apical membrane contains microvilli (Plates 9A, 10, 15A, 16A, 19A, 21A, 23A).

Membrane bound vesicles (mbv) are a consistent feature of all cell types. mbv1 are dense in appearance and are found in particular near the apical membrane (Plates 9A, 10). mbv 2 are light coloured vesicles, more common than mbv, are found in association with - and may be derived from - the Golgi bodies (Plates 10, 11B,18A and B, 20B). They are also to be found near to the apical membrane (Plates 10,21A,23A). It is not known whether mbv1 and 2 fuse with the apical plasma membrane to liberate the microvesicles (eccrine secretion) or not. In addition the rough endoplasmic reticulum may become vesiculated (Plate 11). In some cases the swollen vesiculated cisternae of the rer, contain a material similar in appearance to that in small vesicles found between the microvilli (mv1) (Plates 10,13A and B, 21A). In addition to mv1, microvilli show microvesiculation (mv2) (Plate 10).

Cells of all types may exhibit three additional features:

1. Nuclei may contain crystals (Plates 8A, 11A, 12A, 17A and B). According to Thomas and Gouranton (1978) these are consistent with an iridovirus.
2. Cytoplasmic extrusions through the apical membrane. Some contain organelles such as mitochondria (Plates 13A, 21A) while others contain only a few dilated cisternae of the rer (Plates 13B, many have no discernible structure (Plates 14A and B, 15A).
3. Rosettes of glycogen are found in groups or singly in any position within the cell (Plates 20A, 21B, 22C, 23C).

The above description is generally applicable to the enterocytes of anterior, middle and posterior midguts of fed and starved insects, with certain emphases and exceptions given in Table 1.

Scanning Electron Microscopy (SEM)

An SEM study of the basal surface of the midgut was carried out in order to determine the distribution of the nidi (see Plate 24). A decrease in size of nidi with an increase in density was found from anterior to posterior (see Table 2).

Table 1. Principal features of the fine structure of enterocytes from fed and starved insects

	Fed insects	Starved insects
Anterior midgut	Many type 1 and type 3 cells (Plates 8B, 10, 11B)	Mainly type 2 cells (Plates 8,9A)
	Many mbv 1 and 2 (Plates 10,13A)	Fewer mbv than the fed (Plate 9A)
	Crystals in the nucelus (Plates 8A, 11A)	Few crystals
	Many cytoplasmic extrustions (Plates 13A and B, 14A and B)	Few cytoplasmic extrusions
	No glycogen	No glycogen
Middle midgut	Many type 1 and type 3 cells (Plates 16B, 18A and B)	Mainly type 2 cells (Plates 19A and B, 20A and B)
	Many mbv 2 (Plates 15A, 18B)	Fewer mbv than fed (Plates 19A,20B)
	Crystals in the nucelus (Plates 17A and B)	Few Crystals
	Many cytoplasmic extrusions (Plates 15A and B)	Few cytoplasmic extrusions
	Some rosettes of glycogen (towards posterior end)	Some rosettes of glycogen (towards posterior end (Plates 19A, 20A)

/contd.

Table 1. continued

	Fed insects	Starved insects
Posterior midgut	Mainly type 2 cells (Plates 21C, 22B)	Mainly type 2 cells (Plate 23B)
	Many mbv 2 (Plates 21A, B and C)	Fewer mbv than fed (Plates 23A and C)
	Few crystals	Few crystals
	Many cytoplasmic extrustions (Plate 21A)	Few cytoplasmic extrusions
	Many rosettes of glycogen (Plates 21A, B and C, 22C)	Many rosettes of glycogen (Plates 23A, B and C)

Microvesicles associated with the microvilli (mv 1 and mv 2) are found throughout the midgut of both treatments but are more prevalent in the anterior midgut of fed insects (Plate 10) than elsewhere.

Table 2. Size and density of nidi along the midgut of a fed insect

	Density (nidi/mm^2)			Diameter of nidi in mm		
	mean	SE	N*	mean	SE	N+
Anterior midgut	140	8	4	0.031	0.001	31
Middle midgut	403	123	3	0.027	0.0007	51
Posterior midgut	701	65	2	0.021	0.0005	35

* density determined on this number of low power electron micrographs

+ number of nidi

ABBREVIATIONS

am	apical membrane with microvilli
c	channel between folds of the apical membrane
ce	cytoplasmic extrusions
ch	chromatin
cm	circular muscles
co	connective tissue
cr	crystals
e	enterocyte
el	elongated region of enterocytes - dark in colour
fb	food bolus
g	Golgi body
gl	glycogen
im	immature enterocytes
l	longitudinal muscles
ly	lysosome
m	mitochondrion
mb	multivesicular body
mbv	membrane bound vesicles
mi	microvilli
mt	microtubule
mv	microvesicle
n	nucleus
ni	regenerative nidus
p	expanded proximal region of the enterocyte - light in colour
pm	plasma membrane
pm	peritrophic membrane
rer	rough endoplasmic reticulum

t trachea

v vacuole

vr vesiculated cisternae of the rough endoplasmic reticulum

w short wide enterocytes

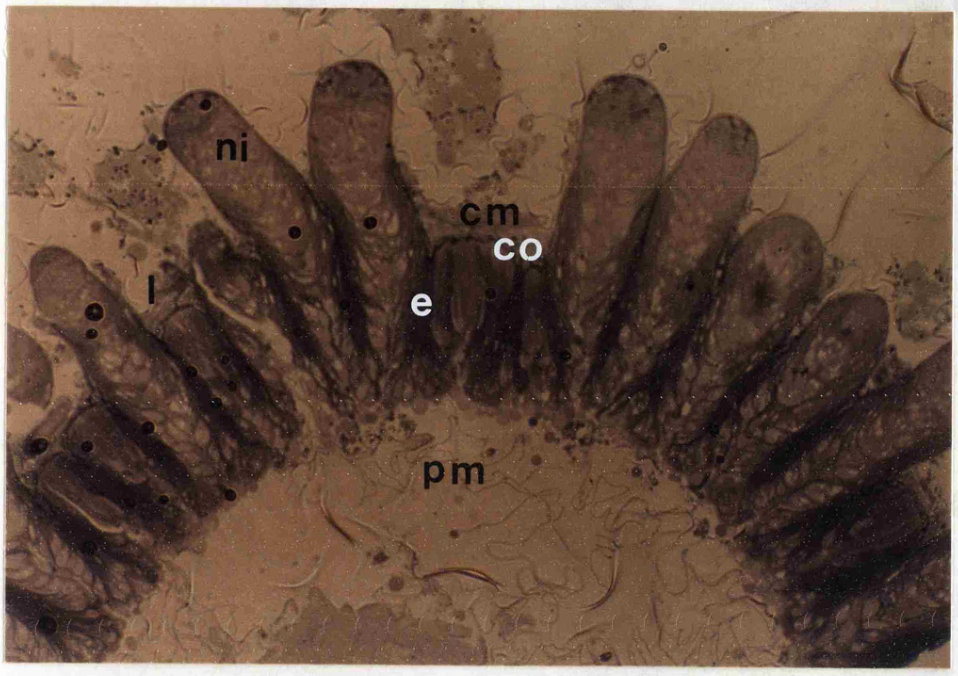
Plate 1. A. T.S. of part of the anterior midgut of a fed insect

The epithelium appears thinner than that of the starved insect (see Plate 1B) because it is stretched by the presence of food in the gut.

B. T.S. of part of the anterior midgut of a starved insect. X160.

Note: e, enterocyte, ni, regenerative nidus; co, connective tissue; cm circular muscles; l, longitudinal muscles; pm, peritrophic membrane. X160.

A



B

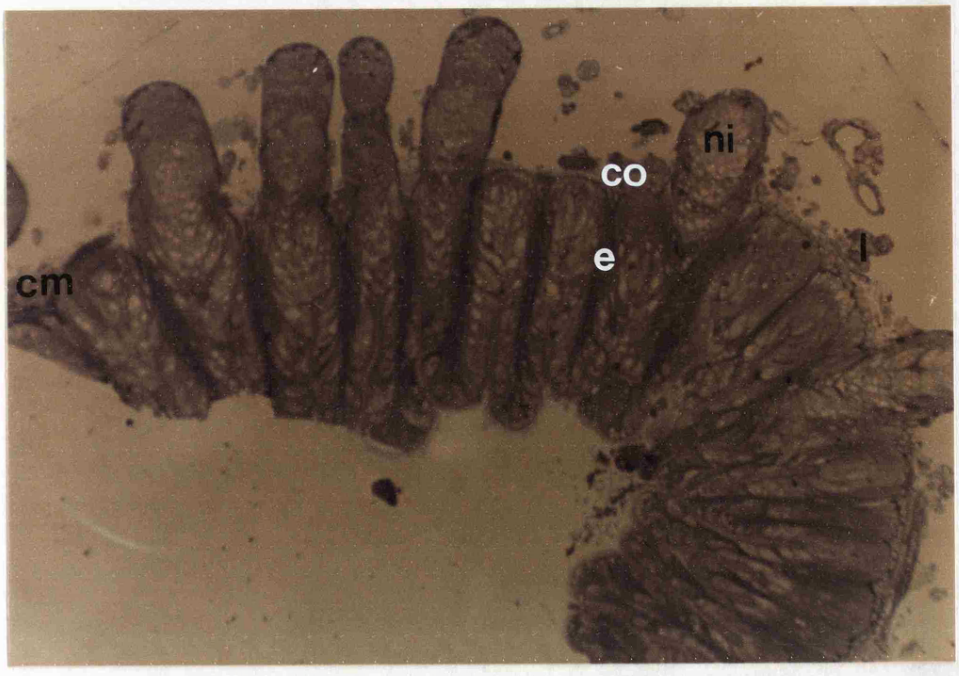


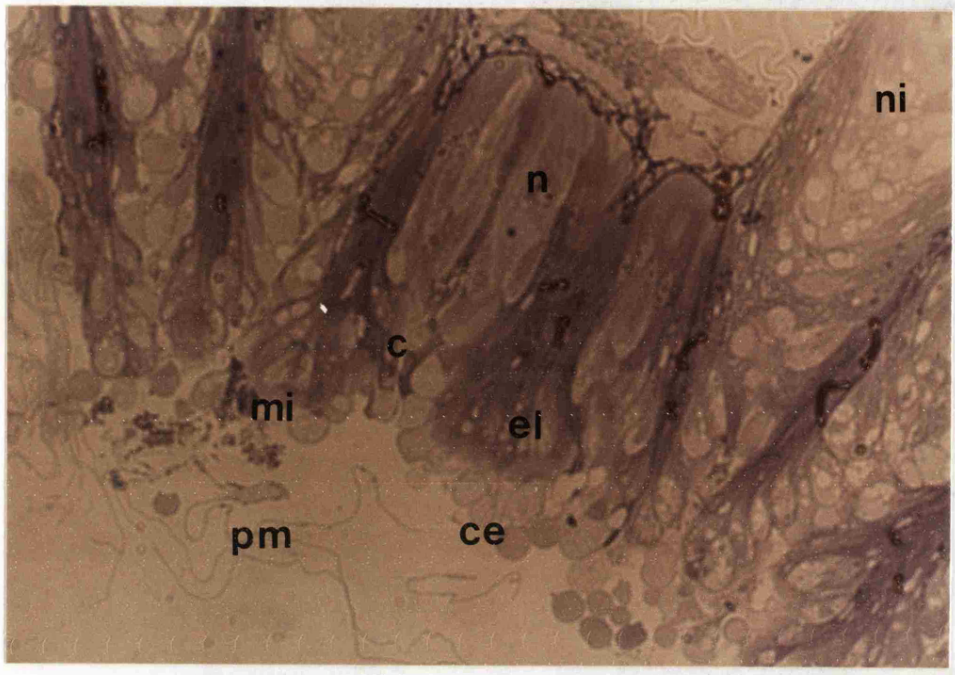
Plate 2. A. T.S. of part of the anterior midgut of a fed insect, showing the enterocytes

Note: c, channel between folds of the apical membrane, mi, microvilli; n, nucleus; el, elongated region of enterocytes; ce, cytoplasmic extrusions; pm, peritrophic membrane; ni, nidus with small embryonic cells. X300.

B. T.S. of part of the anterior midgut of a starved insect showing the enterocytes.

Note: c, channel between folds in the apical membrane, m, microvilli; im, immature enterocytes; n, nucleus; el, elongated region of enterocyte - dark in colour; p, expanded proximal region of the enterocyte-light in colour; v, vacuoles. X300.

A



B

Plate 3. High power light micrograph of part of the anterior midgut of a fed insect.
Not all the enterocytes are thin and elongated (el), some are short and relatively wide (w) X630.

Note: n, nucleus; ce, cytoplasmic extrusion; pm, peritrophic membrane

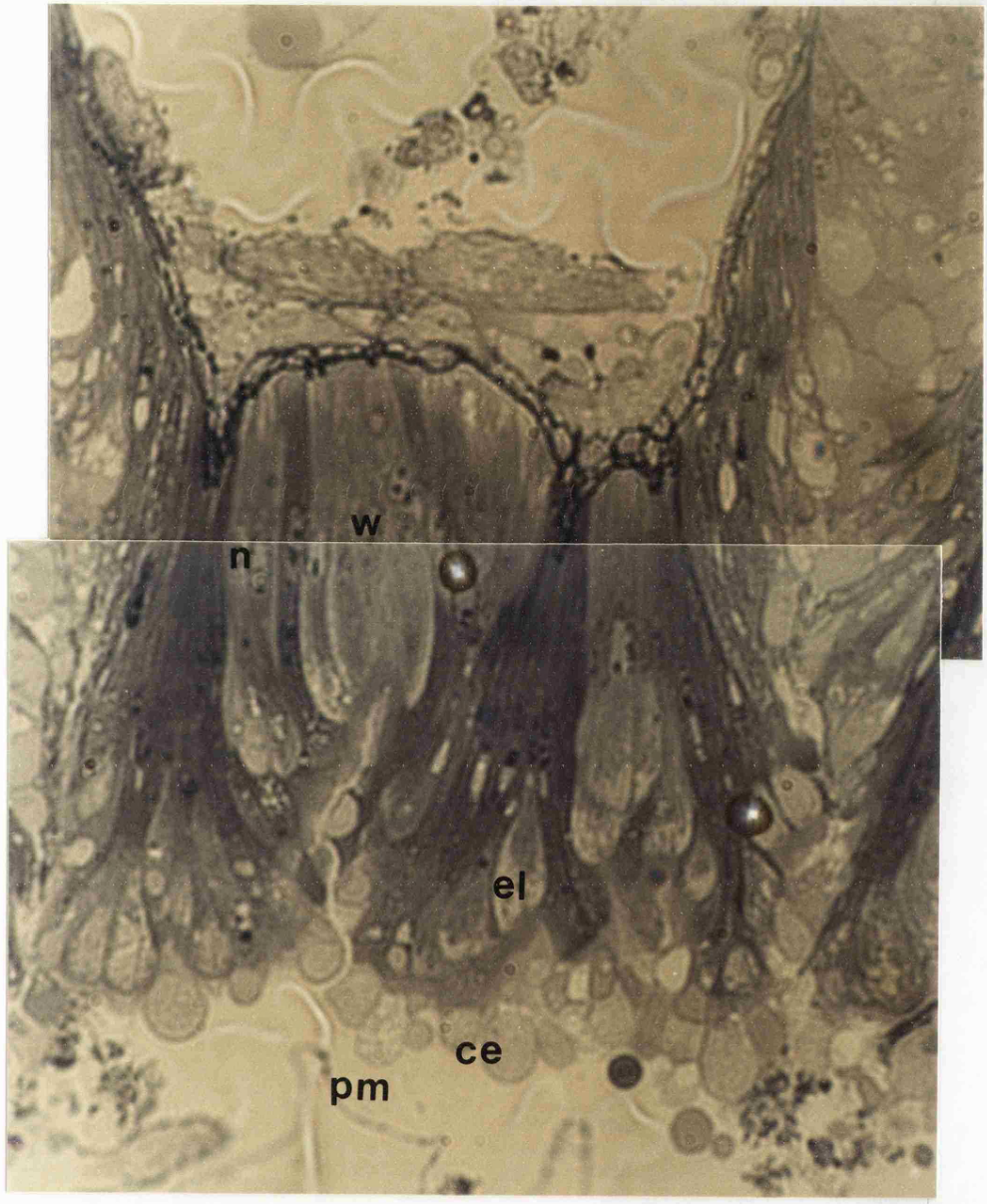

w
n
el
ce
pm

Plate 4. High power light micrograph of part of the anterior midgut of a starved insect.
Not all the enterocytes are thin and elongated (el), some are short and relatively wide (w). X630.

Note: n, nucleus, c. channel between folds of the apical membrane; m, microvilli, ct, connective tissue; cm, circular muscles; l, longitudinal muscle, v, vacuole.

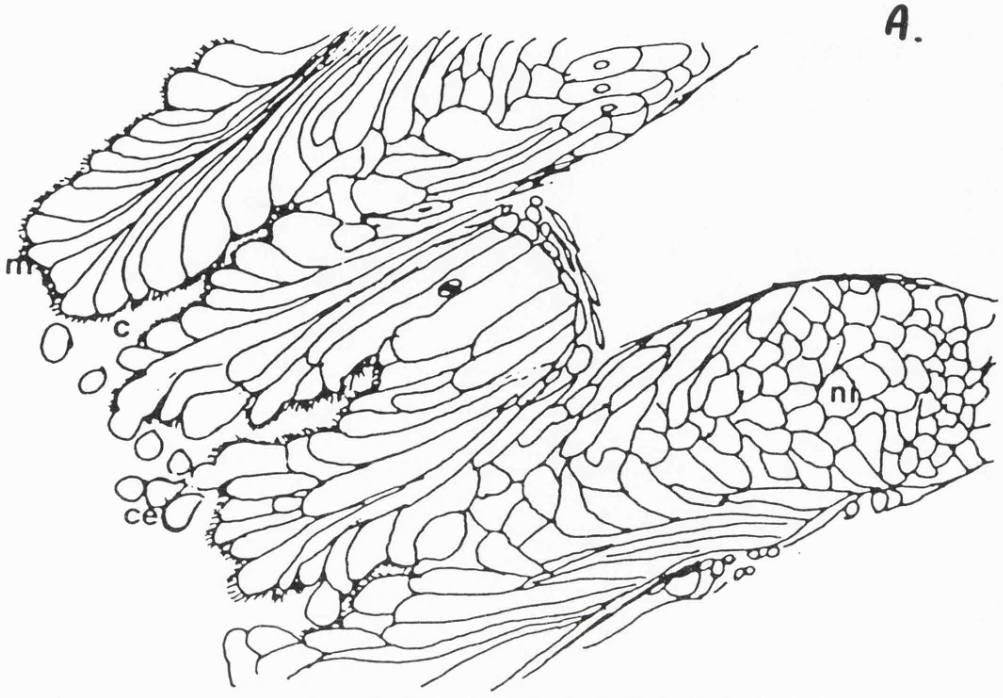

cm
ct
n
w
el
c
v
m

Fig. 1. A. Diagrammatic representation of part of the anterior midgut of a fed insect

Note: c, channel between folds of the apical membrane; ni, embryonic cells in the nidus; ce, cytoplasmic extrusions, mi, microvilli.

B. Diagrammatic representation of part of the anterior midgut of a ~~fed~~ starved insect

The next generation of secretory cells grow down from the nidus to replace the old enterocytes. En route they appear to fan out both within the plane of the section and at right-angles to it, as a result some are cut almost in T.S. (f). It seems that as cells grow down either side of a channel they become constricted (c), giving them an elongated appearance. X300.


A.
m
c
ce
ml

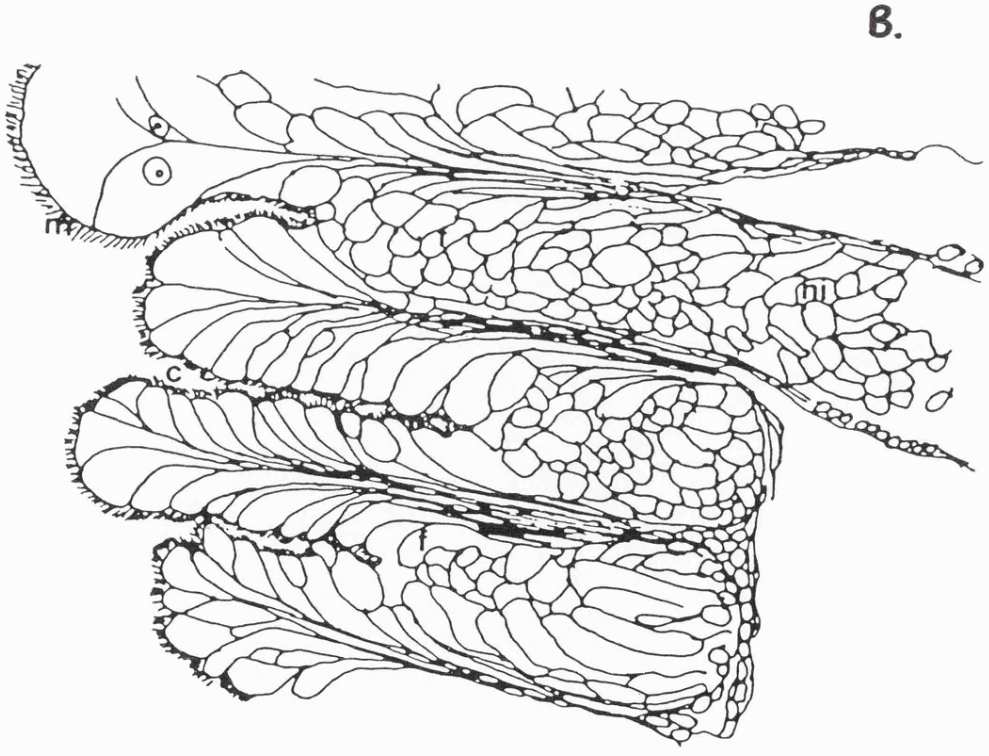

B.
m
ml
c
f

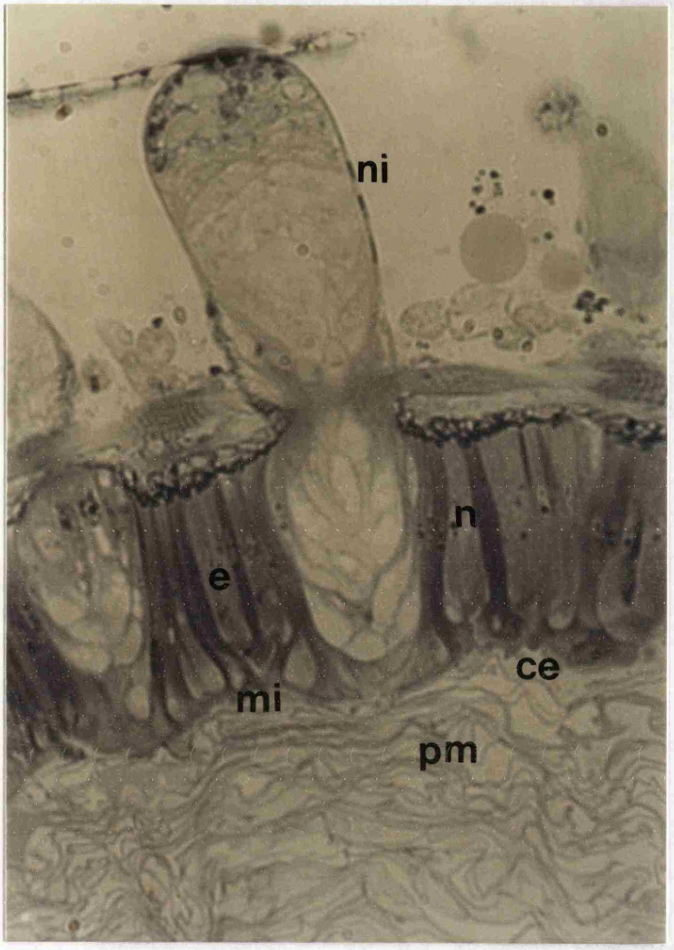
Plate 5. A. Middle midgut of a fed insect

Unlike in the anterior gut (see Plate 2) the enterocytes are mostly short and relatively wide and the apical membrane is not thrown into folds. Cytoplasmic extrusions are present, but they are not as extensive as in the anterior part of the fed midgut (see Plate 2). X 220.

B. Middle midgut of a starved insect

Similar in appearance to the comparable region in the fed insect (see Plate 5A) with the exceptions that the peritrophic membrane is sparse and there are few cytoplasmic extrusions. X220

n, nucleus, ni, regenerative nidus; pm, peritrophic membrane; ce, cytoplasmic extrusion; mi, microvilli; e, enterocyte


ni
n
e
ce
mi
pm

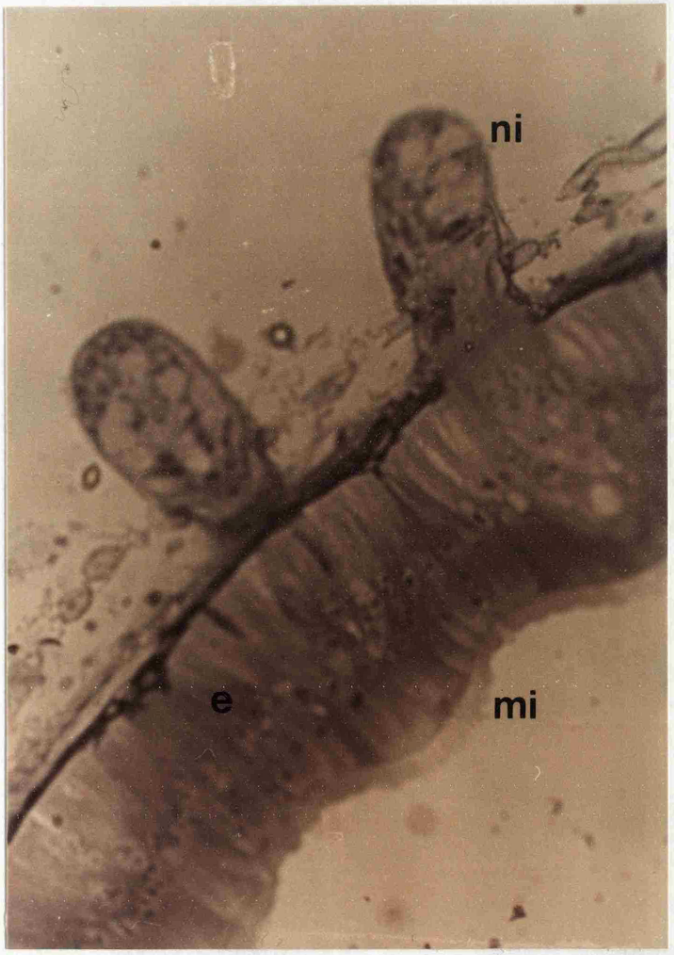

ni
e
mi

Plate 6. A. Posterior midgut of a fed insect

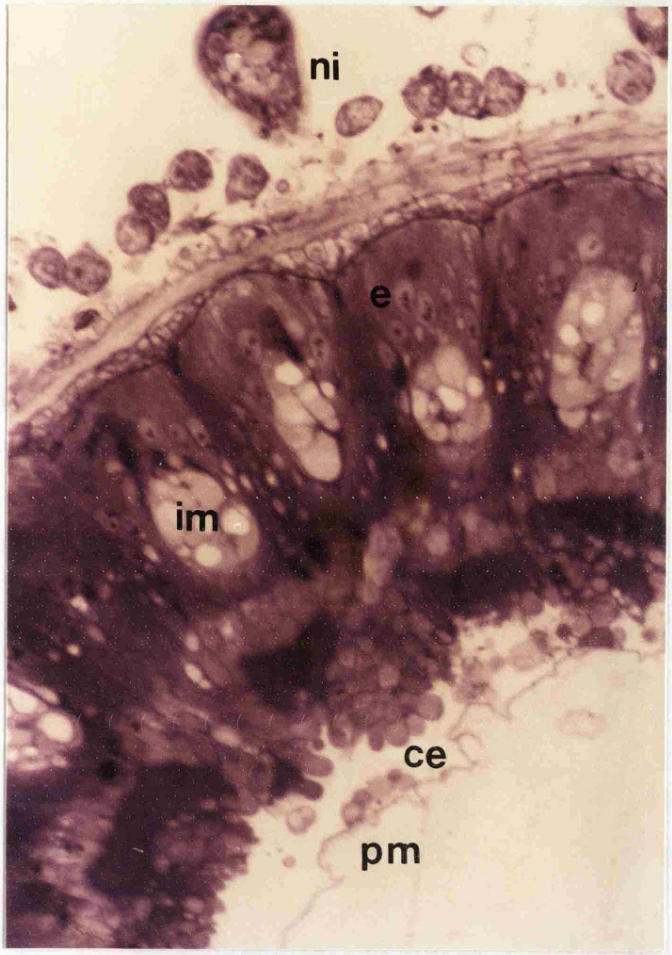
The section depicted in this micrograph has cut obliquely through areas of the epithelium containing "immature" enterocytes which have recently descended from nidi, these appear as lightly stained cells. There are many cytoplasmic extrusions (cf. Plates 2 and 5A). X220

B. Posterior midgut of a starved insect

Note the folding of the apical membrane and the absence of cytoplasmic extrusions (cf. Plates 2B and 5B) X220

el, elongated region of enterocytes; c, channel between folds of the apical membrane; ni, nidus; mi, microvilli; ce, cytoplasmic extrusions; im, immature enterocytes

A



B

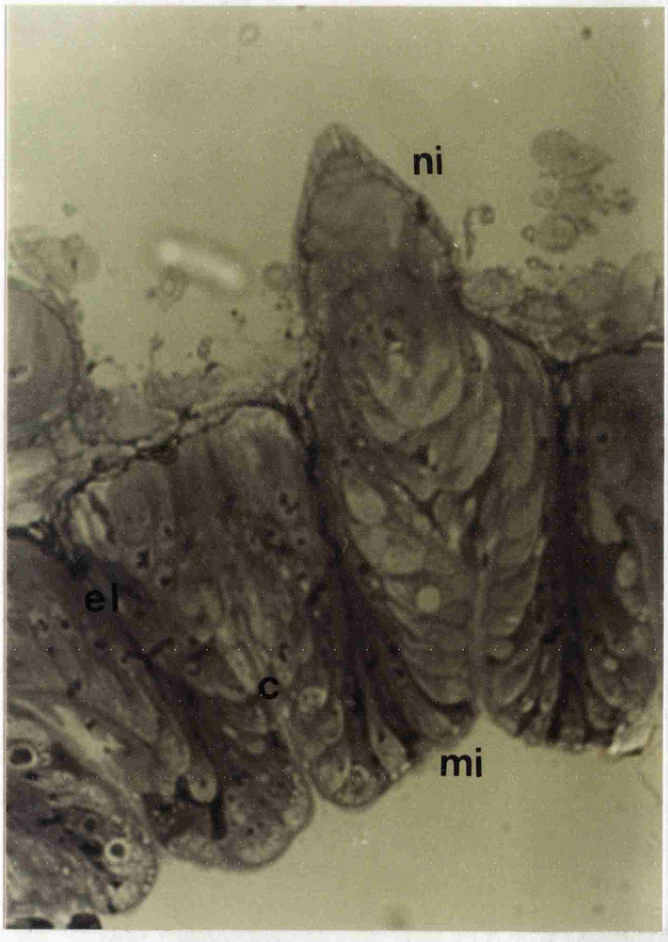


Plate 7. A. Low power electron micrograph of the anterior midgut epithelium of a starved insect

The apical membrane is thrown into folds. Many of the enterocytes are cut in T.S. as they fan outwards from the nidus (cl) (see Fig. 1); others have been sectioned obliquely (almost in L.S.), have nuclei and appear long and thin (c2). Some cells (x) have few observable structures apart from some membrane bound vesicles (mbv) ("type 1" cells) possibly derived from rough endoplasmic reticulum, and mitochondria. T.S. X 4,599

Note: am, apical membrane with microvilli; n, nucleus, m, mitochondrion; v, vacuole

B. Low power electron micrograph of the anterior midgut epithelium of a fed insect

Similar in general organisation to the epithelium of the starved insect (see A above). "x" cells contained few observable structures apart from mitochondria and membrane bound vesicles (type 1 cell). "y" enterocytes are characterised by extensive dilated cisternae of the rough endoplasmic reticulum (rer 1) (substantially "type 3" cells).

Note: m, mitochondrion, n, nucleus; m, apical membrane with microvilli. T.S. X 3,373

A

B

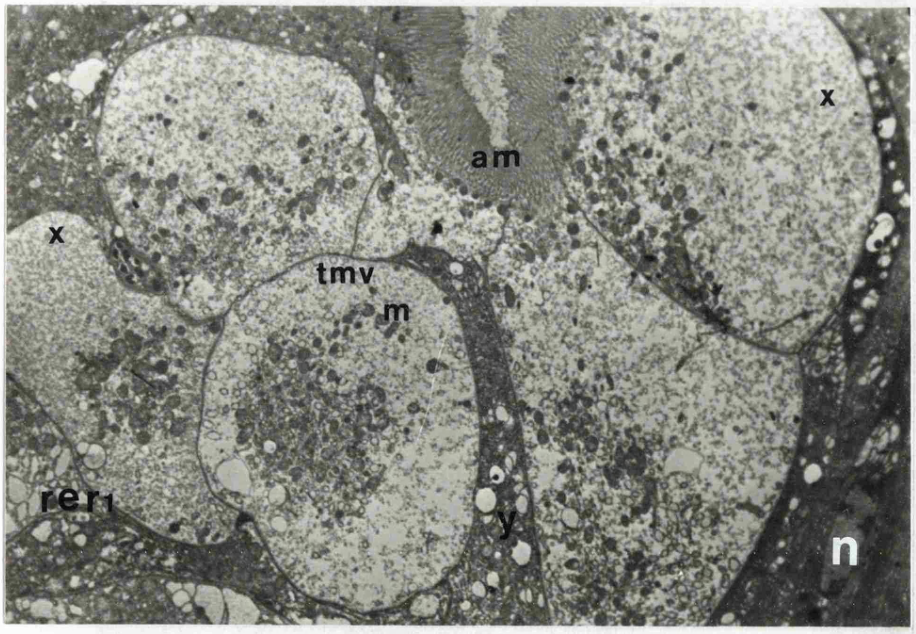


Plate 8. A. Electron micrograph of enterocytes from a starved insect. In this section of a T.S. of the anterior midgut the cells have been cut almost completely in L.S., and it can be seen how the cells fan out towards the apical membrane. Although there is much rough endoplasmic reticulum it is not conspicuous (substantially "type 2" cells). X3390

Note: am, apical membrane with microvilli, n, nucleus; m, mitochondrion; pm, plasma membrane; ly, lysosome; rer2, non dilated cisternae of the rough endoplasmic reticulum; cr, crystal; ch, chromatin, pm, plasma membrane

B. Electron micrograph of enterocytes from a fed insect. The enterocytes in this micrograph have been cut obliquely. Rough endoplasmic reticulum dominates the cytoplasm of the cells, many of the cisternae are highly dilated ("type 3" cells) and it is difficult to tell them apart from elements of Golgi apparatus. X 4435.

Note: n, nucleus; m, mitochondrion; rerl, dilated cisternae of the rough endoplasmic reticulum

A

B

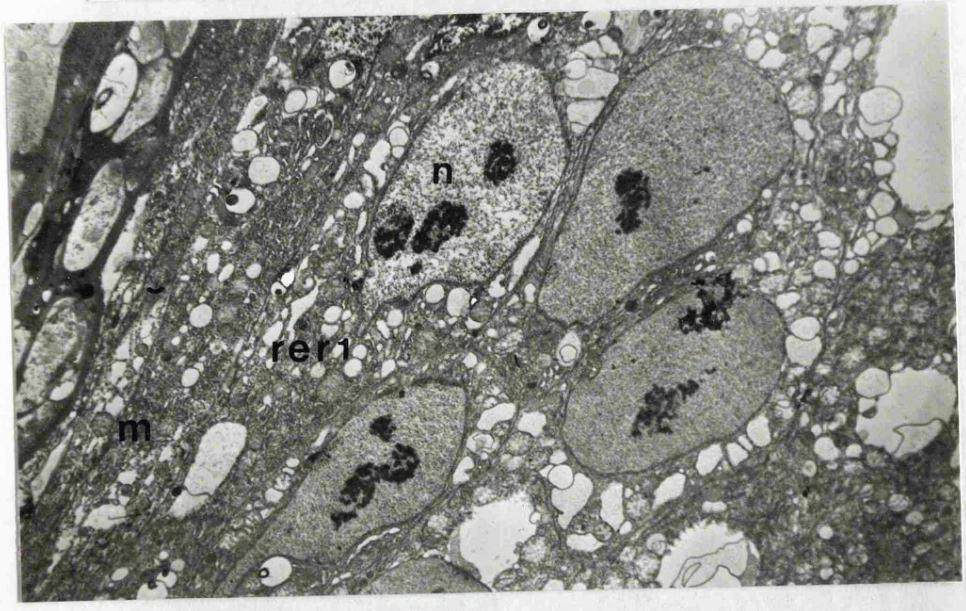


Plate 9. A. Low power electron micrograph montage of a T.S. of the anterior midgut of a starved insect.

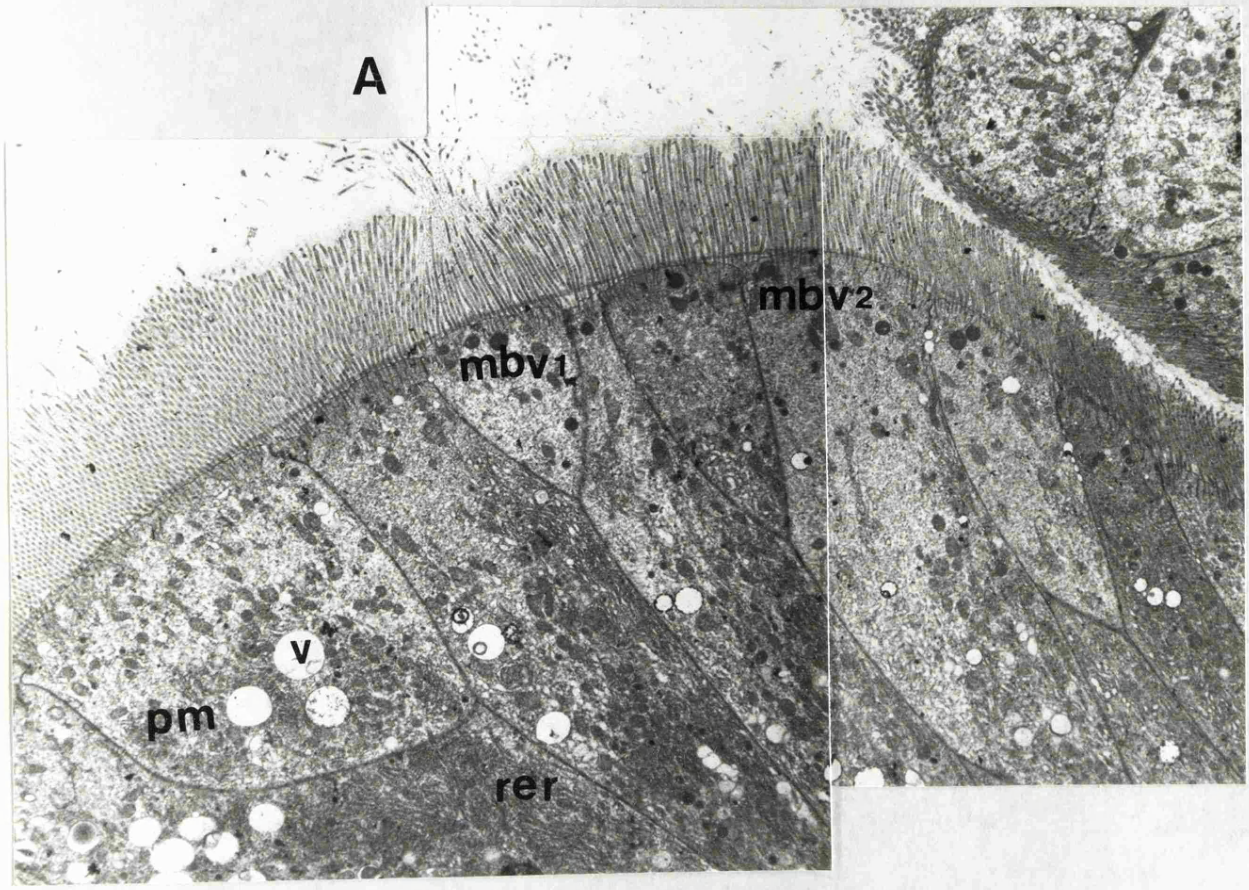
Area adjacent to the apical membrane has few organelles apart from mitochondria and membrane bound vesicles. Some large vacuoles are present which may represent lipid droplets. X3630

Note: am, apical membrane with microvilli; rer, rough endoplasmic reticulum; v, vacuole (lipid droplet); mbv1, dense membrane bound vesicle; mbv2, light membrane bound vesicle, pm, plasma membrane

B. Electron micrograph of a T.S. of enterocytes from a starved insect.

Note the moderately dilated cisternae and many Golgi bodies (substantially "type 2" cells)

Note: rerl, dilated cisternae of the rough endoplasmic reticulum; g, Golgi body, m, mitochondrion. X 8820.


A
mbv2
mbv1
v
pm
rer

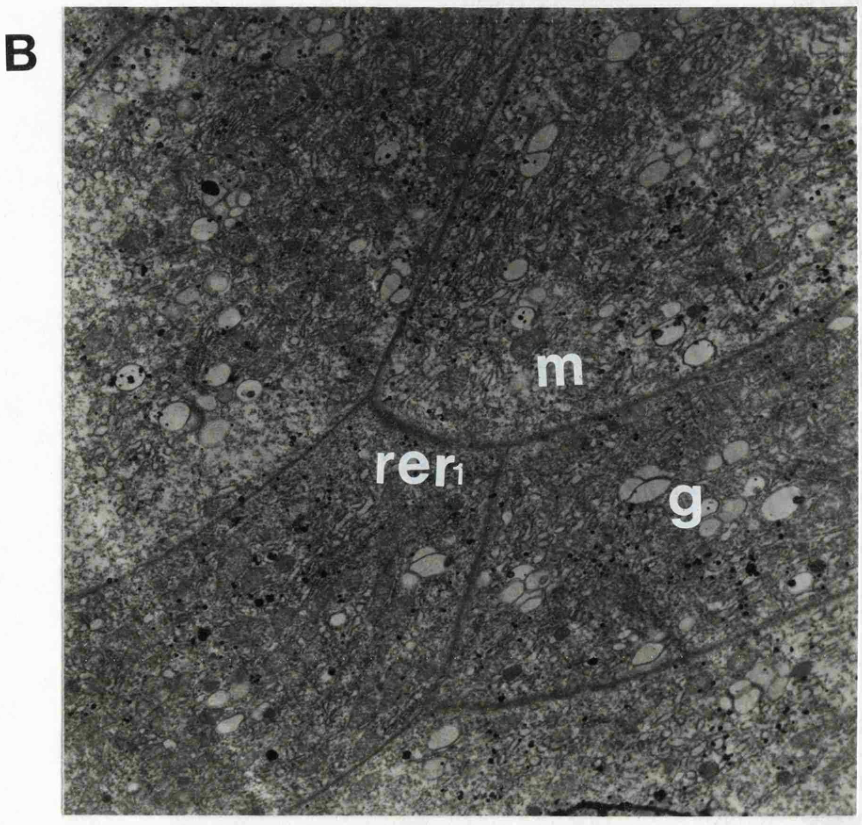

B
m
rer1
g

Plate 10. Apex of a number of anterior midgut enterocytes from a fed insect. Cytoplasm contains membrane bound vesicles, some are electron dense (mbv1 see Plate 9) others are lighter in appearance (mbv 2). The rough endoplasmic reticulum takes the form of vesicles (vr) or consists of dilated cisternae (rer 1) ("type 3" cells). The elements of the rer contain a material similar in appearance to that in the small vesicles present between the microvilli. Small vesicles also seem to be forming at the tips of some of the microvilli.

Note: am, apical membrane with microvilli; mv1, chains of microvesicles between microvilli; mv2, micro vesicles forming at the tips of microvilli; mbv1, dense membrane bound vesicles; mbv2, light membrane bound vesicles; pm, peritrophic membrane; m, mitochondrion. X 8510

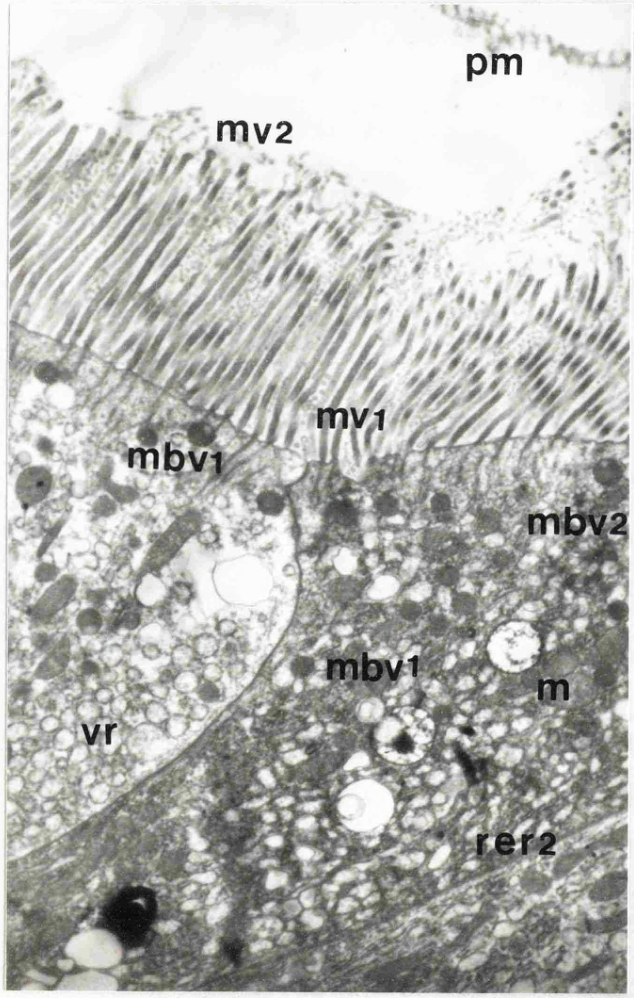

pm
mv2
mv1
mbv1
mbv2
mbv1
m
vr
rer2

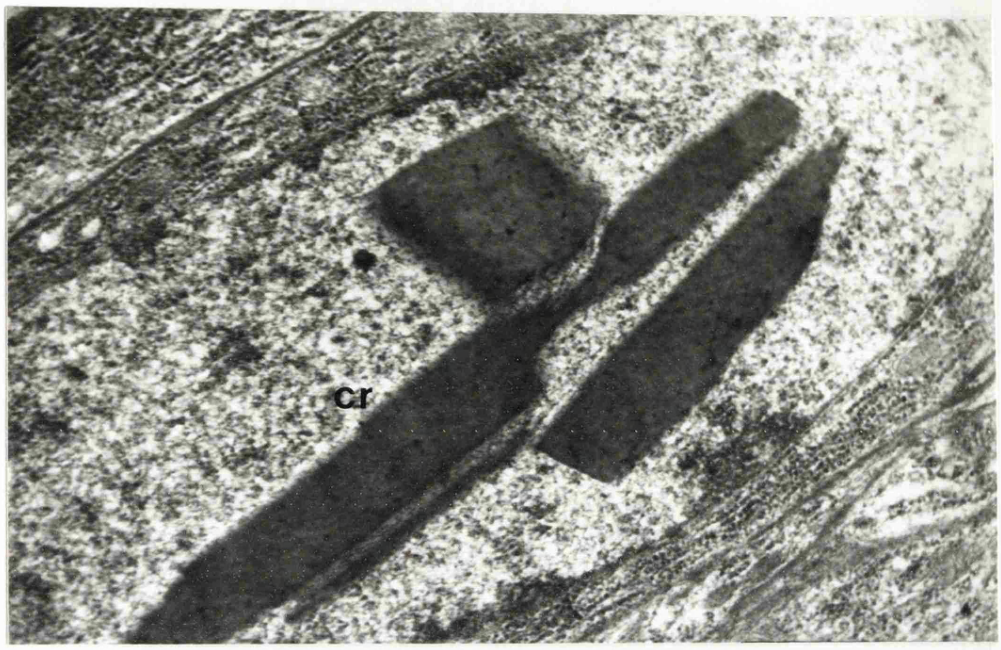
Plate 11. A. High power electron micrograph of the nucleus of an anterior midgut enterocyte from a fed insect. It shows the osmiophilic crystals that Thomas and Gouranton (1978) described as iridovirus particles. Although these were often found in the nuclei of enterocytes from fed insects, they were only infrequently present in cells of starved insects.

Note: cr, crystal. X26980

B. Electron micrograph of anterior midgut enterocytes from a fed insect.

Note: rer1, dilated cisternae of the rough endoplasmic reticulum; rer2, flattened cisternae of the rough endoplasmic reticulum; mbv 2, light coloured membrane bound vesicles (see Plate 10); g, Golgi body (mainly "type 3" cells). X5564

A



B

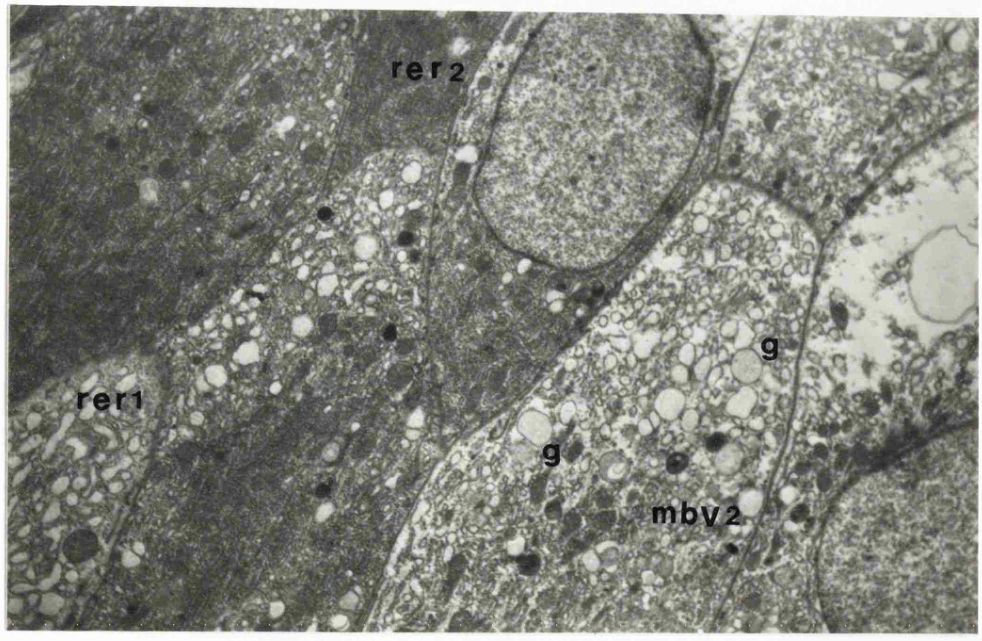


Plate 12. A. L.S. of anterior midgut enterocytes from fed insect. "type 2" cells. X 8208

Note: rer, rough endoplasmic reticulum; n, nucleus; cr, crystal, ch, chromatin

B. Electron micrograph of an anterior midgut enterocyte from a starved insect.
The cell is full of rough endoplasmic reticulum which is slightly dilated (substantially "type 3" cell). Microvesicles appear to be forming from the cisternae of the rough endoplasmic reticulum.

Note: rerl, rough endoplasmic reticulum,; v, vesicles forming from rer cisternae.
X14318

A

B

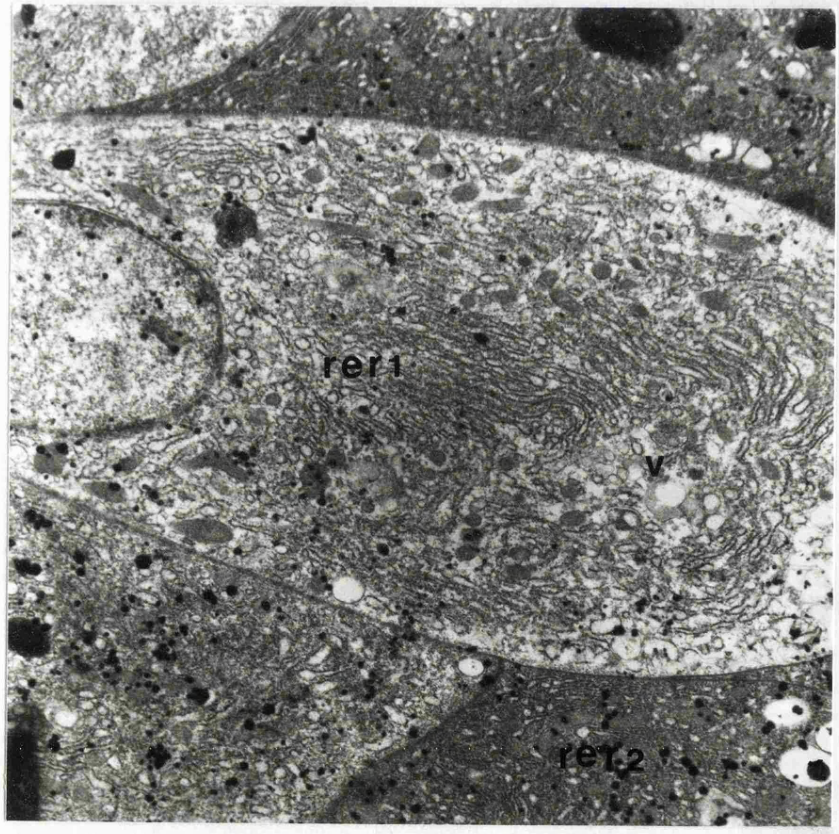


Plate 13. A. Cytoplasmic extrusion in the anterior midgut of a fed insect.

The cells show dilation of the cisternae of the rough endoplasmic reticulum which contains a flocculent material (rer 1). The extrusion contains a number of cellular organelles. X8208.

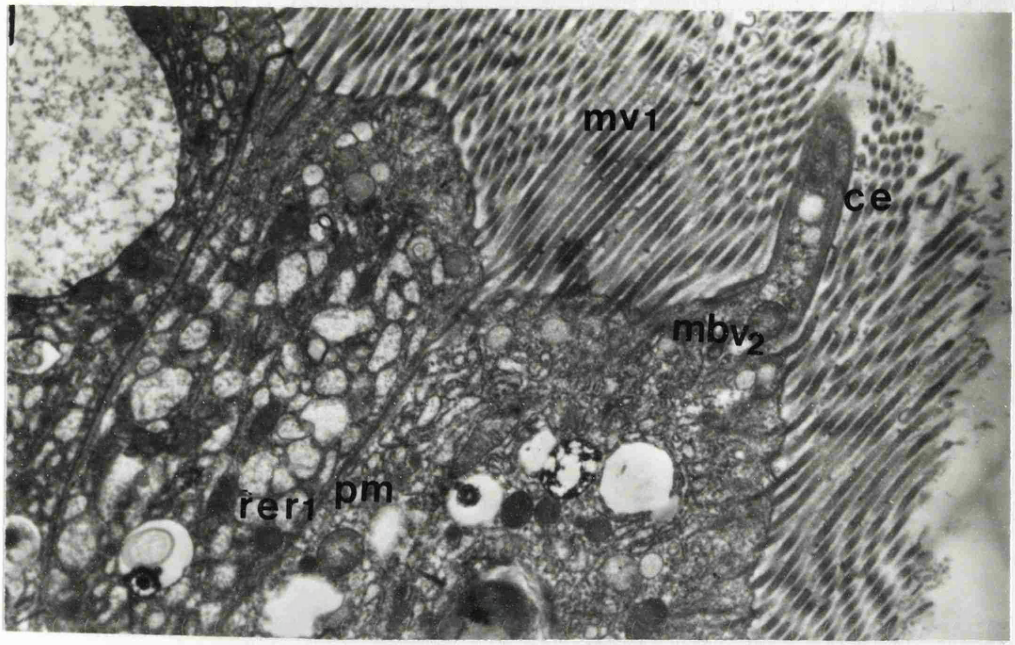
Note: rerl, dilated cisternae of the rough endoplasmic reticulum,; pm, plasma membrane, mbv2, membrane bound vesicles; ce, extrusion n, nucleus; mvl, microvesicles between the microvilli.

B. Cytoplasmic extrusion in an anterior midgut enterocyte from a fed insect. X 5417.

Extrusion contains only dilated cisternae of the rough endoplasmic reticulum.

Note: ce, extrusion; mvl, microvesicles between the microvilli.

A



B

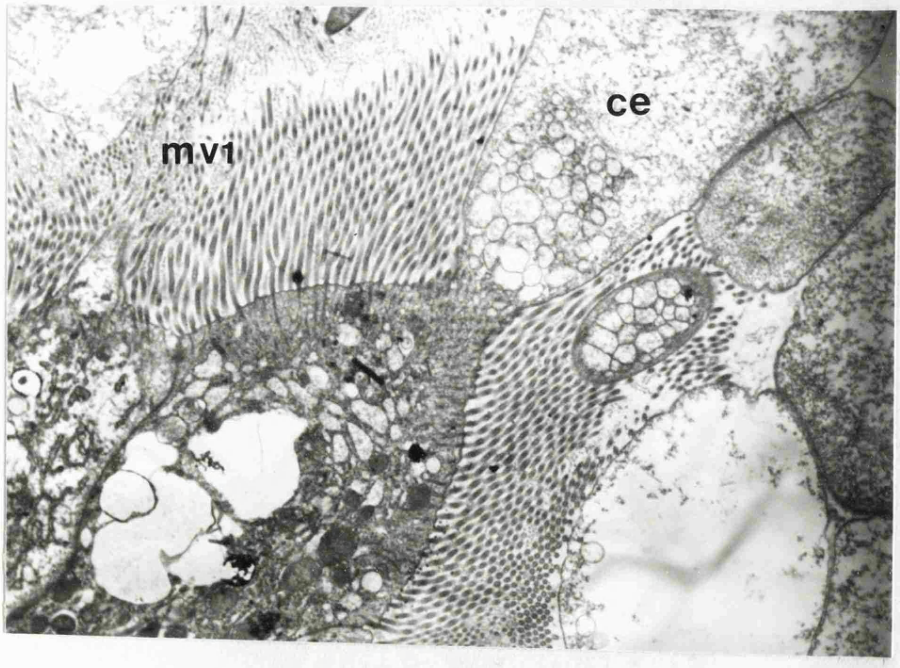


Plate 14. A. Cytoplasmic extrusion in an anterior midgut enterocyte from a fed insect. Extrusion contains no discernible structures.

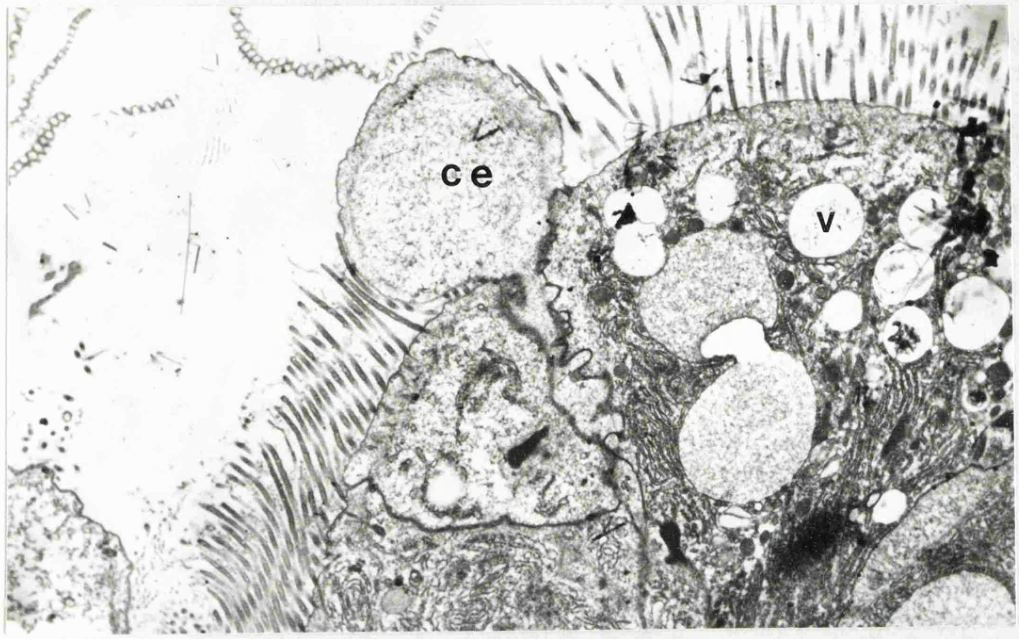
Note: ce, extrusion; pm, peritrophic membrane; v, vacuole (lipid droplet) X 5685

B. Extrusions from the anterior midgut of a fed insect

A number of extrusions on the surface of enterocytes. Some are detached from the cells others are still joined. None of them contain any discernible structures.

Note: ce, extrusions. X2739

A



B

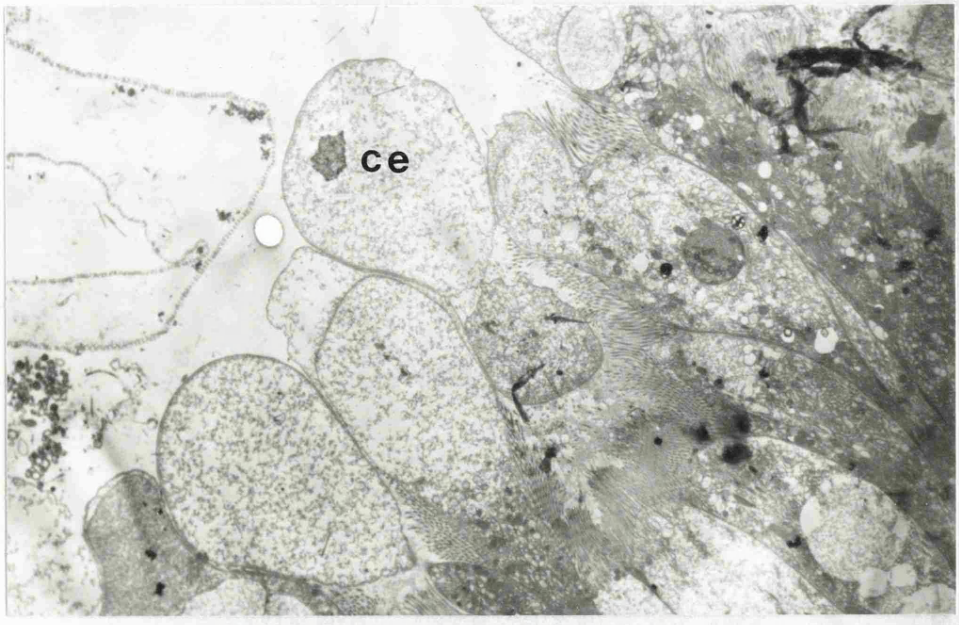


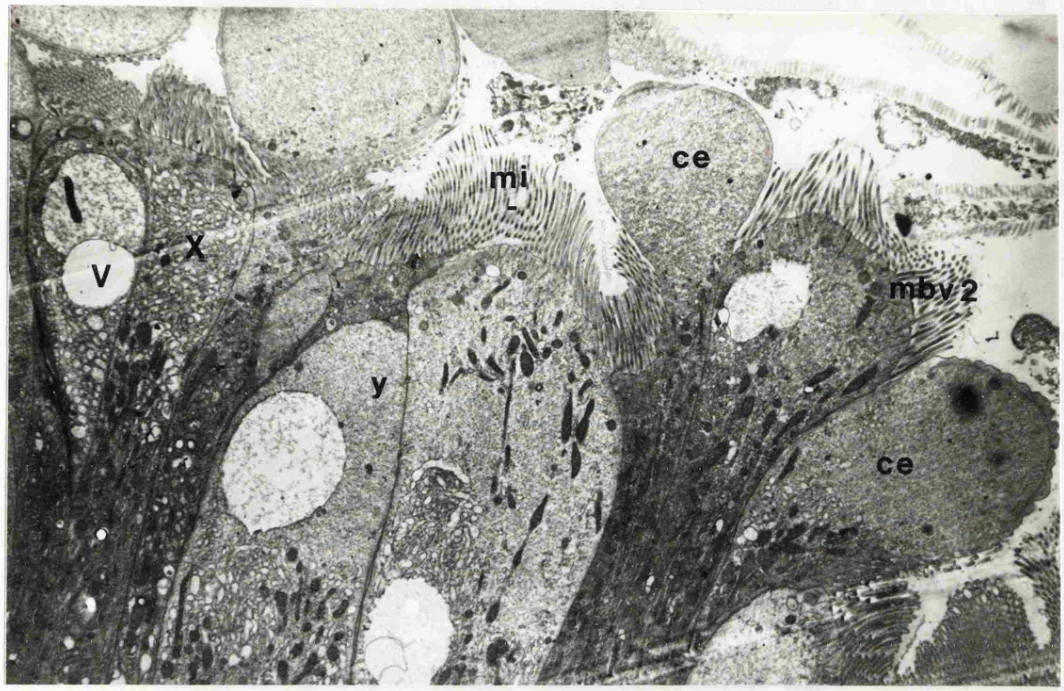
Plate 15. Low power electron micrographs of enterocytes from the middle midgut of fed insects.

A. Cytoplasmic extrusions again in evidence; similar in appearance to those in anterior fed midgut (see Plates 13 and 14). Cells exhibiting areas corresponding to several cell types; x, "type 2" and "type 3"; y, "type 1" and "type 3" . TS X3731

B. Cytoplasmic extrusions again in evidence; similar in appearance to those in anterior fed midgut (see Plates 13 and 14). Cells exhibiting areas corresponding to several cell types; y, substantially "type 1"; x, substantially "type 2". TS x 1865

Note: ce, cytoplasmic extrusions; mi, microvilli, mbv2, light coloured membrane bound vesicles.

A



B

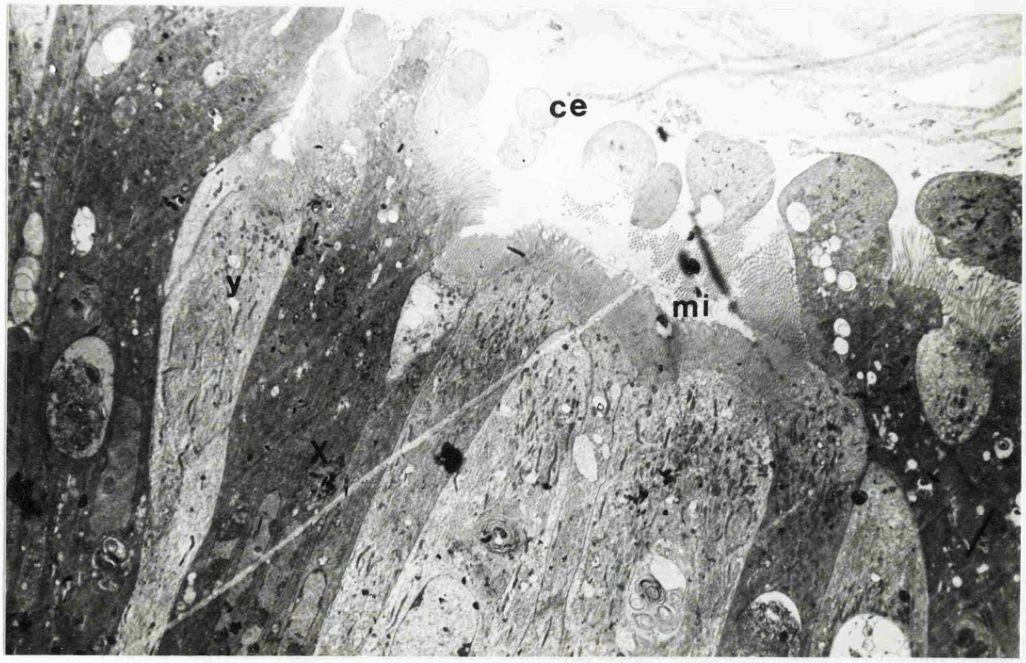


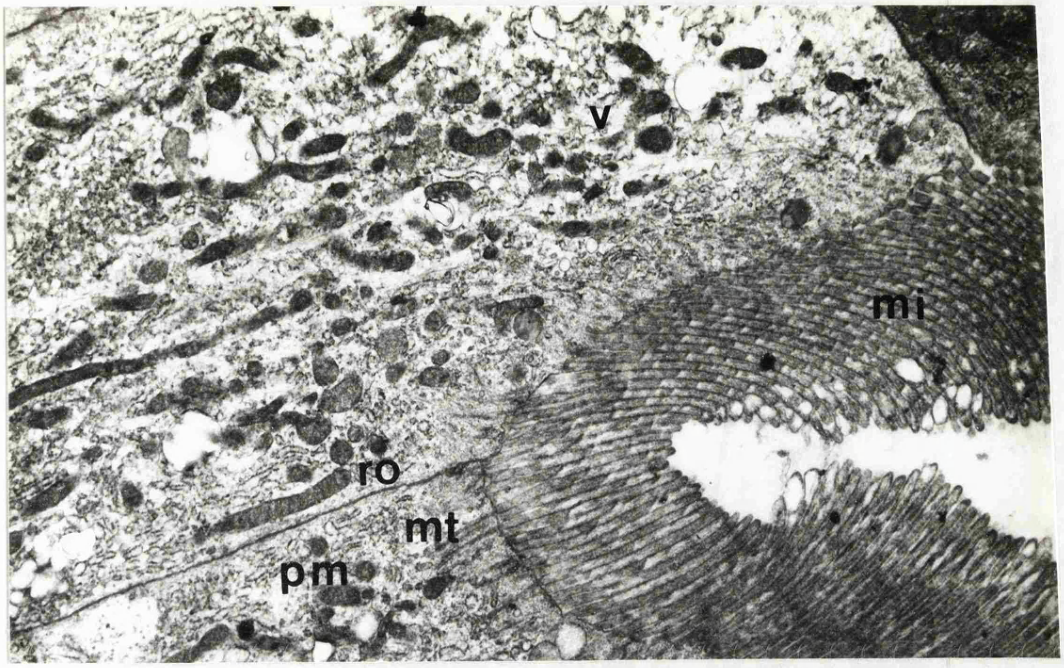
Plate 16. Apical membrane of the middle midgut from fed insects

A. These enterocytes have many mitochondria and the microvilli have swollen tips. The cytoplasm is light in appearance and contains many small vacuoles. Some elements of the rough endoplasmic reticulum are dilated. X 9836

B. Y, substantially "type 1" cell; x, substantially "type 2) cell. Chains of small microvesicles are found between and possibly derived from the microvilli (see Plate 10). X9327

Note: rerl, dilated cysternae of the rough endoplasmic reticulum; rer 2, non dilated cysternae; mi, microvilli; mvl,microvesicles; mt, microtubule; pm, plasma membrane, v. vesicle

A



B

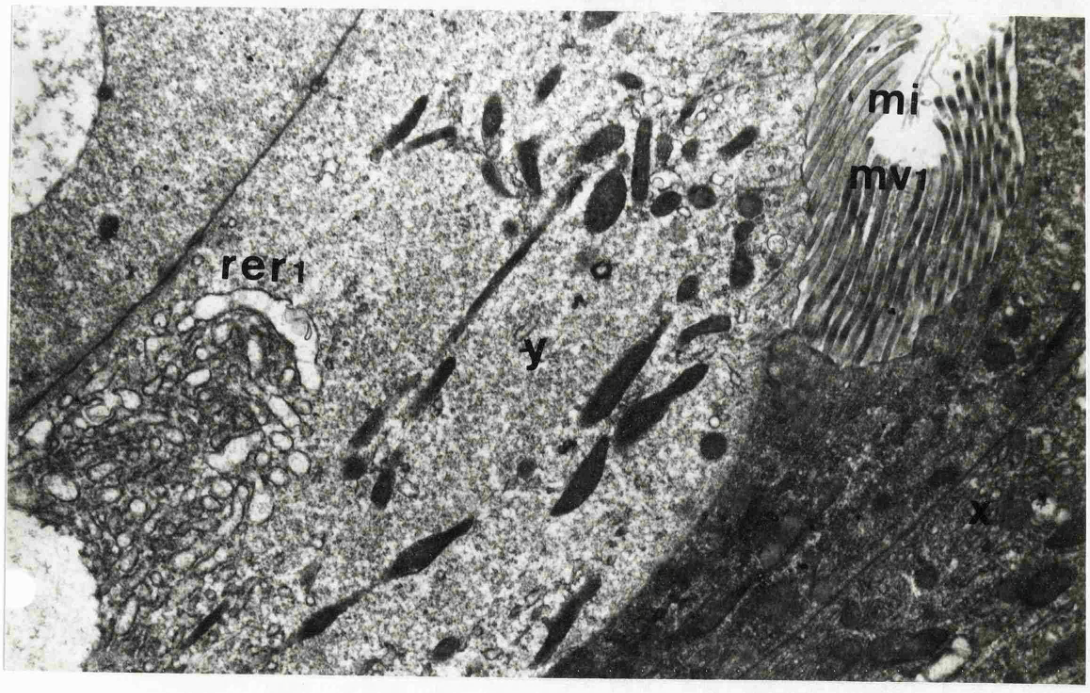


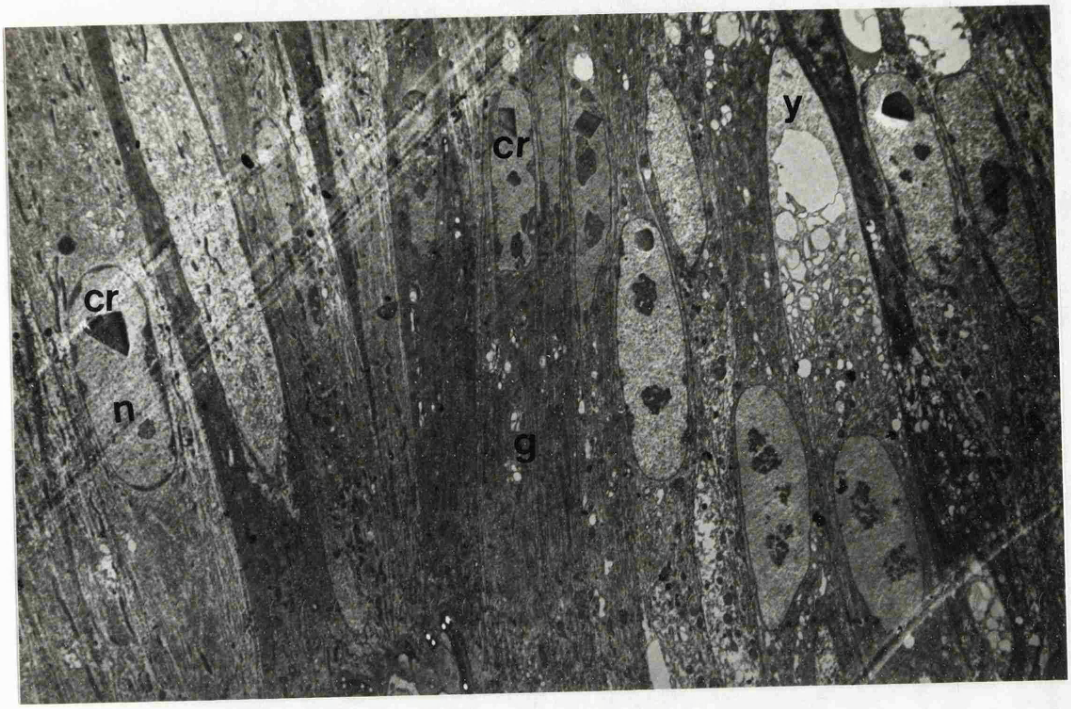
Plate 17. Enterocytes from the middle midgut of a fed insect

A. low power electron micrograph showing the parallel arrangement of cells in this region of the gut. Many of the nuclei contain crystals (see Plate 11). Most of the cells correspond to "type 2", but "y" in particular illustrates all three conditions ("types 1, 2 and 3") X2425

B. The centrally placed enterocyte in this micrograph is characterised by slight dilation and vacuolation of the rer. X12079

Note: cr , crystal, n, nucleus; g, Golgi body; ch, chromatin; rerl, dilated cisternae of the rough endoplasmic reticulum; rer 2, non dilated cisternae; v, vacuoles apparently derived from the rer

A



B

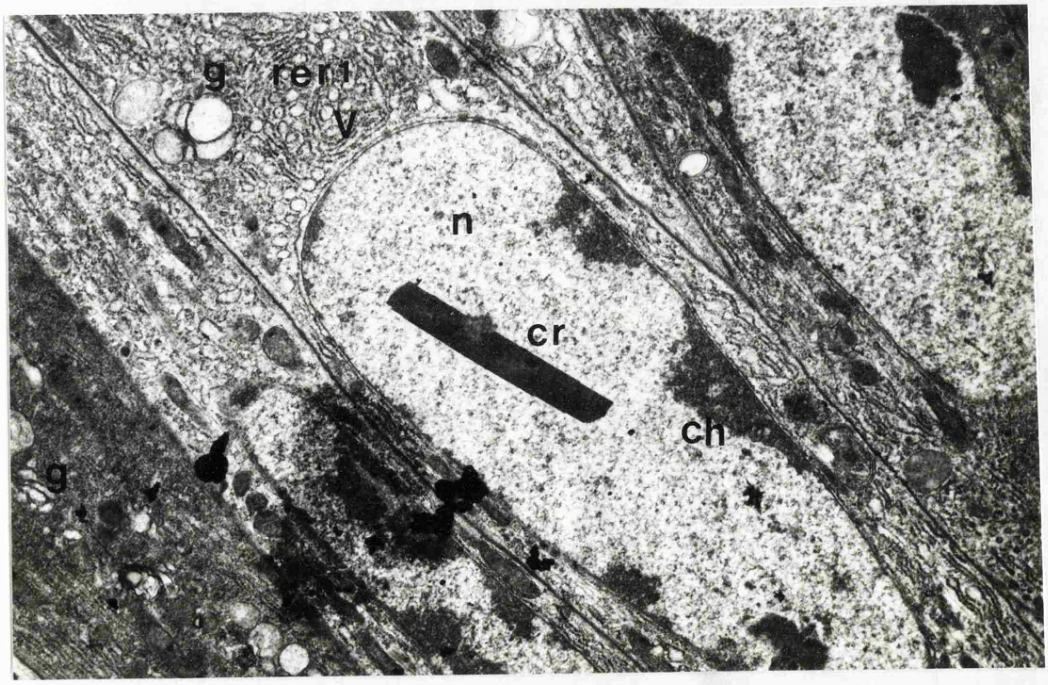


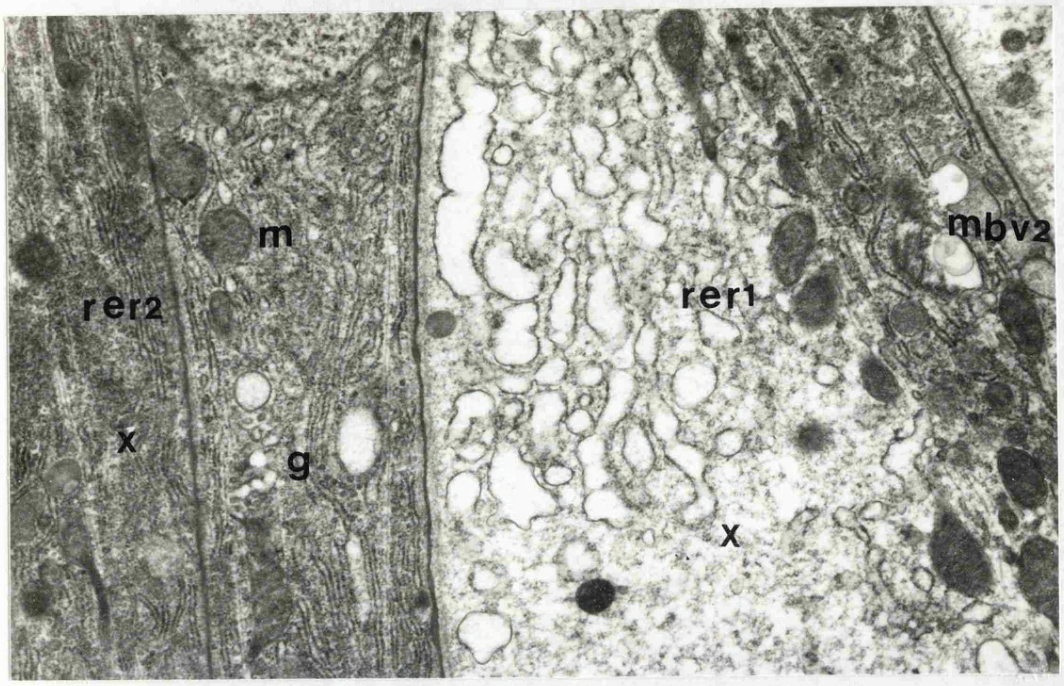
Plate 18. Enterocytes from the middle midgut of a fed insect.

A. x, "type 2" cell; y, substantially a "type 3" cell. X15191

B. Many prominent Golgi bodies in these enterocytes, some are associated with membrane bound vesicles (mbv 2, see eg. Plates 10, 11B, 13A) ("type 3" cells) X 12079

Note: g, Golgi body; rer 1, slightly dilated cisternae of the rough endoplasmic reticulum; mbv1, light coloured membrane bound vesicle; m, mitochondrion.

A



B

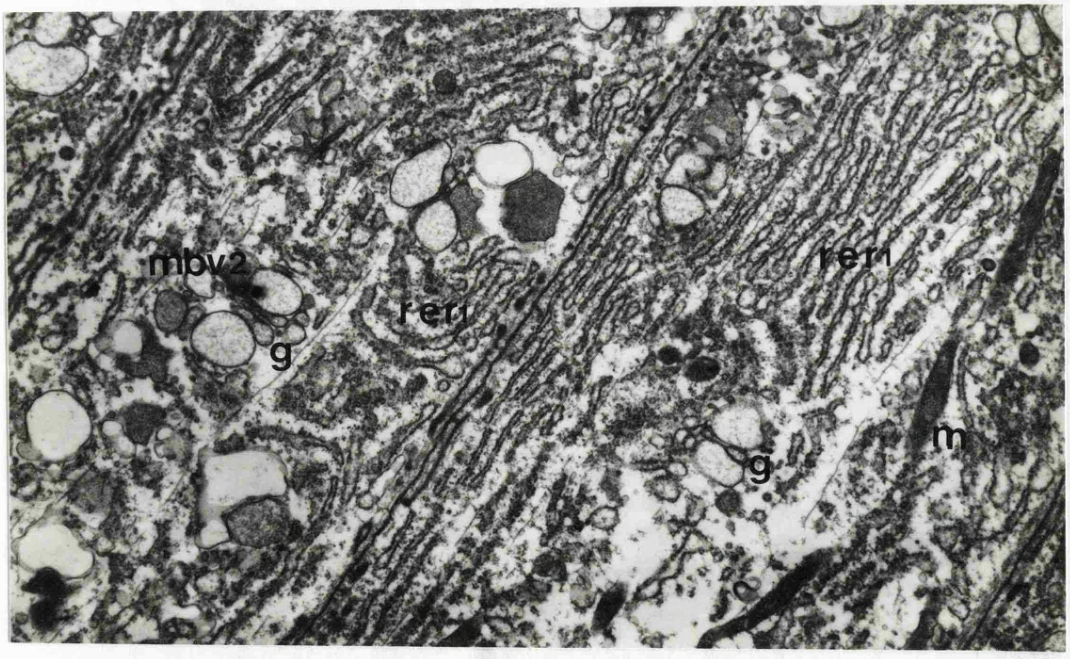


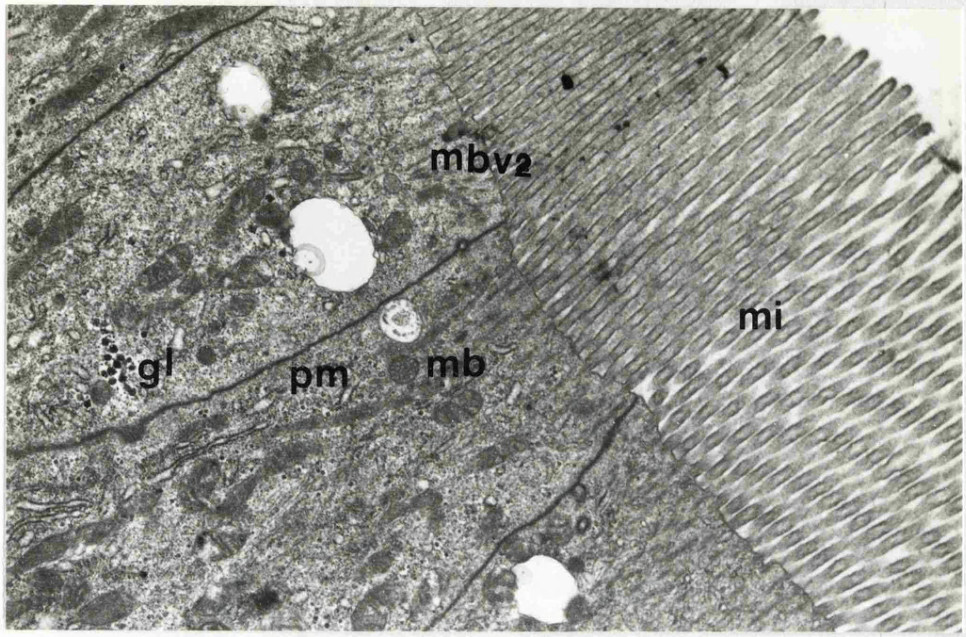
Plate 19. Enterocytes from the middle midgut of a starved insect. Both micrographs show "type 2" cells

A. Apical membrane with microvilli. X 10612

B. Nucleus has no crystals. X 10482

Note: gl, rosettes of glycogen; mi, microvilli, n, nucleus; g, Golgi body; rer2, non dilated cisternae of the rough endoplasmic reticulum; mb, multivesicular body.

A



B

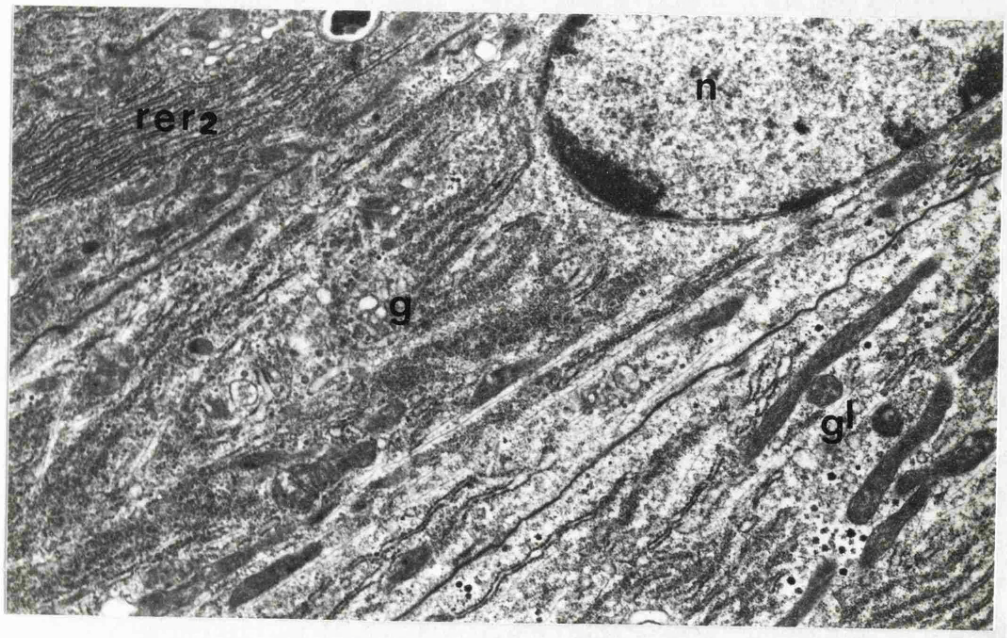


Plate 20. Electron micrographs of enterocytes from the middle midgut of a starved insect
All 3 plates depict "type 2" cells. A number of prominent Golgi bodies are present

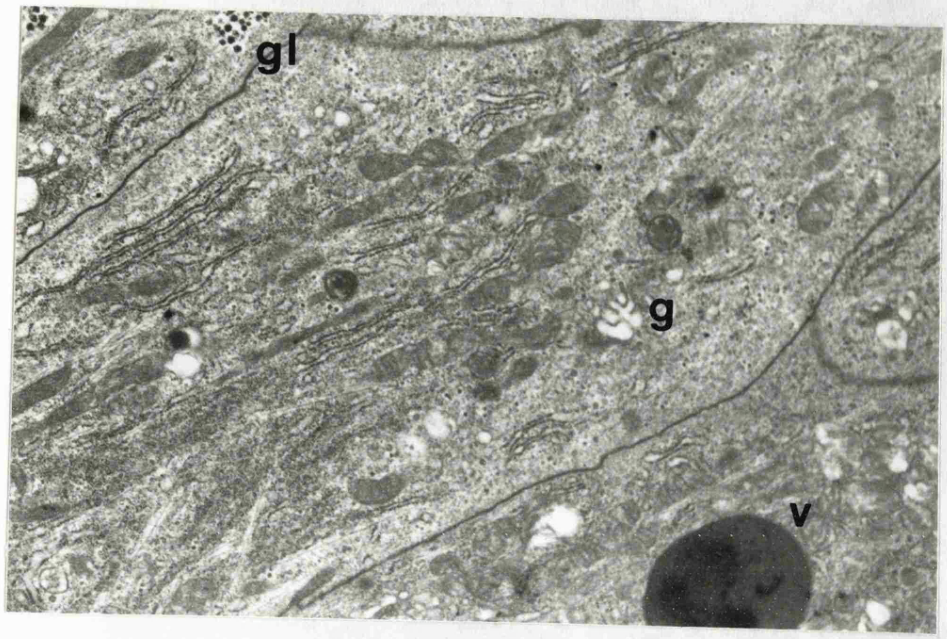
A X11259

B X24700

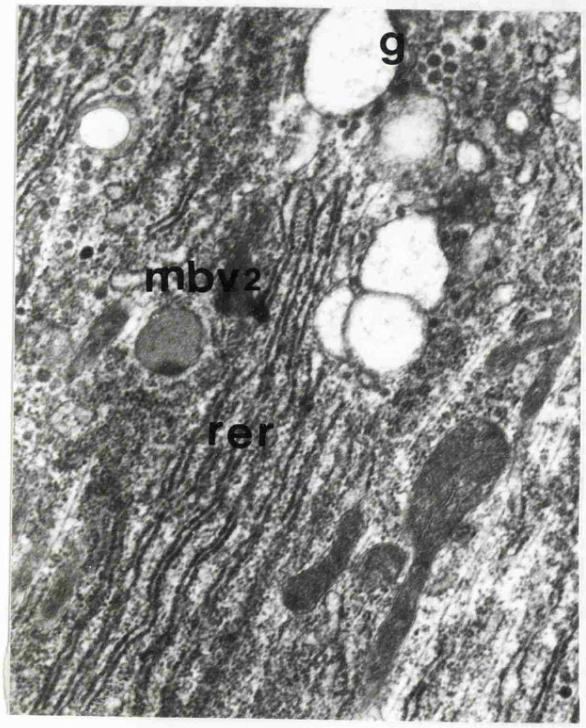
C X53706

g, Golgi body; rer 2, non dilated cisternae of the rough endoplasmic reticulum; gl, glycogen; v, osmiophilic droplet; mbv2, light membrane bound vesicle

A



B



C

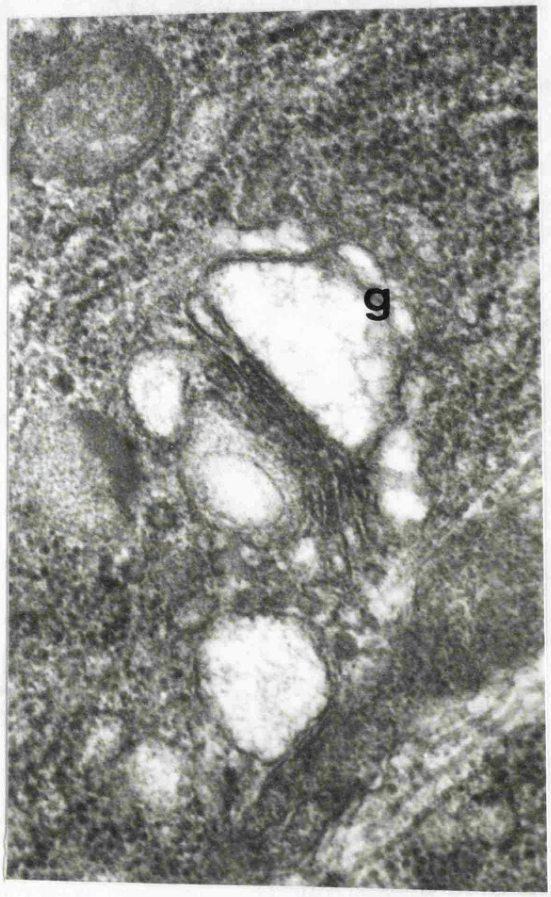


Plate 21. Enterocytes from the posterior midgut of fed insect

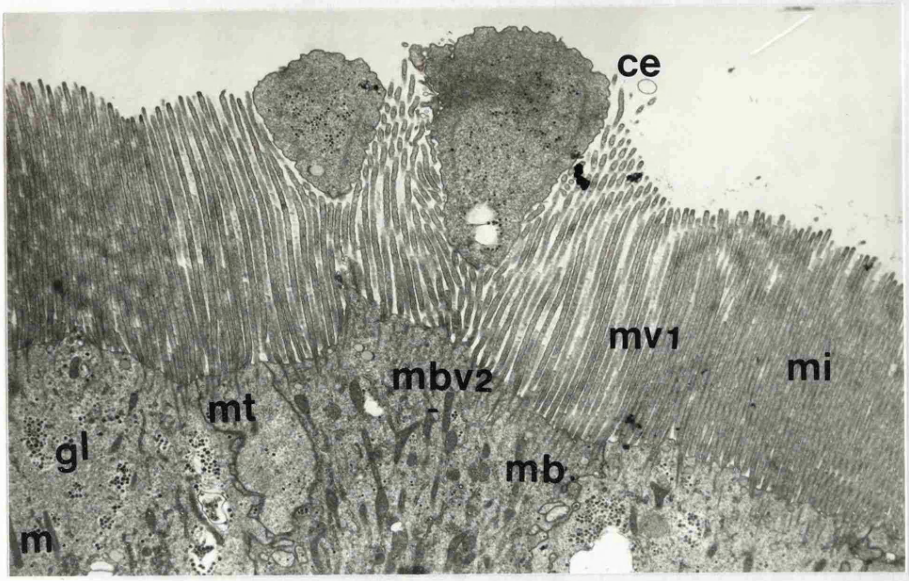
A. Apical membrane with cytoplasmic extrusions and microvilli. Note the many membrane bound vesicles X5094

B. Apical membrane with cytoplasmic extrusions and microvilli. Note the many membrane bound vesicles X10,171

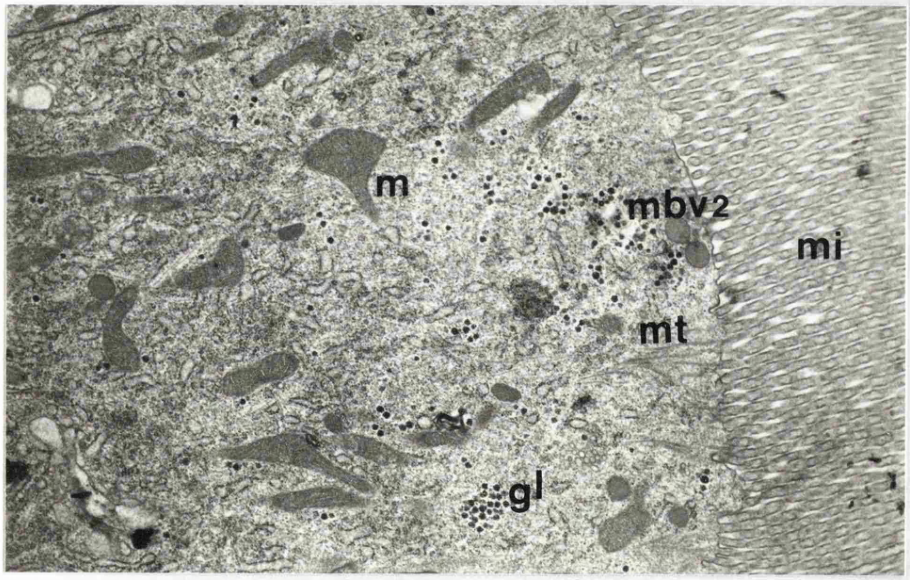
C. Many Golgi bodies and membrane bound vesicles ("type 2" cells) X 7,692

Note: g, Golgi body; mbv2, light coloured membrane bound vesicles; gl, glycogen; mt, microtubules; mi, microvilli; m, mitochondrion; mb, multivesicular body; ce, cytoplasmic extrusion

A



B



C

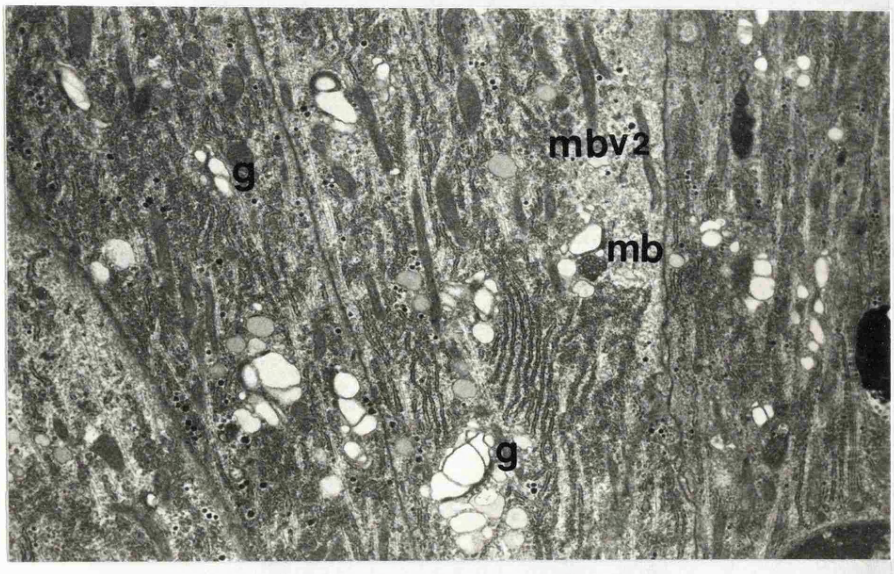


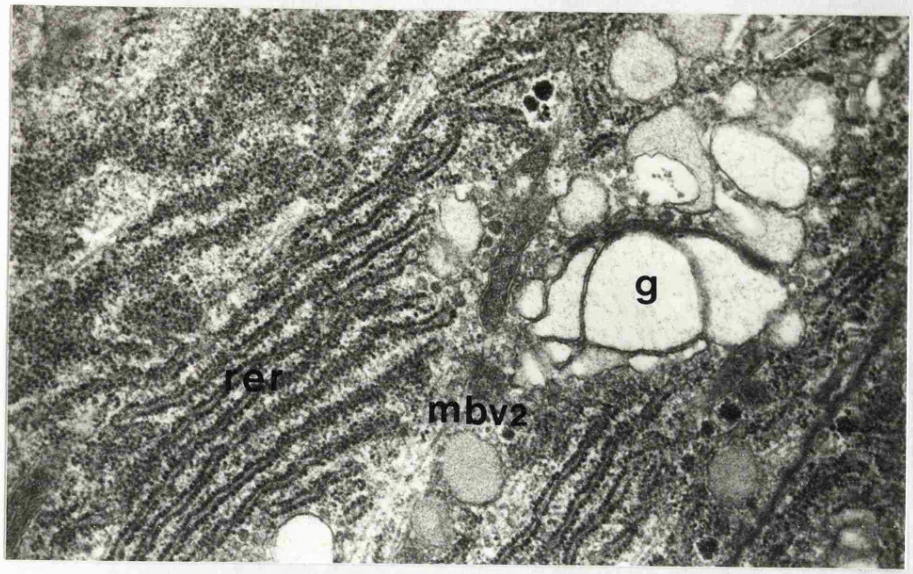
Plate 22. Enterocytes from the posterior midgut of a fed insect

A. High powered electron micrograph of a Golgi body and associated membrane bound vesicles X 24,615

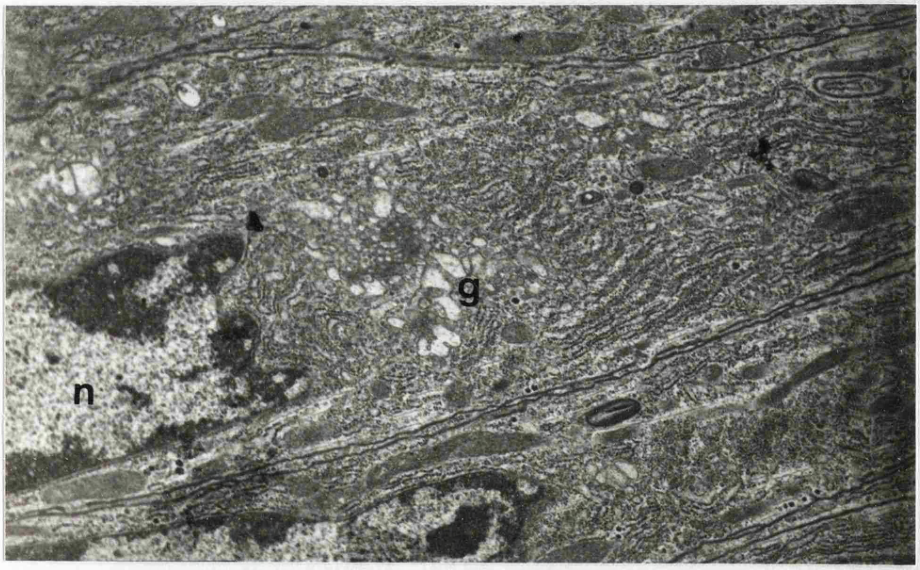
B. "type 2" cells, note the nucleus has no crystal(s) X 10,171

C. High powered electron micrograph of rosettes of glycogen. X24,615.

A



B



C

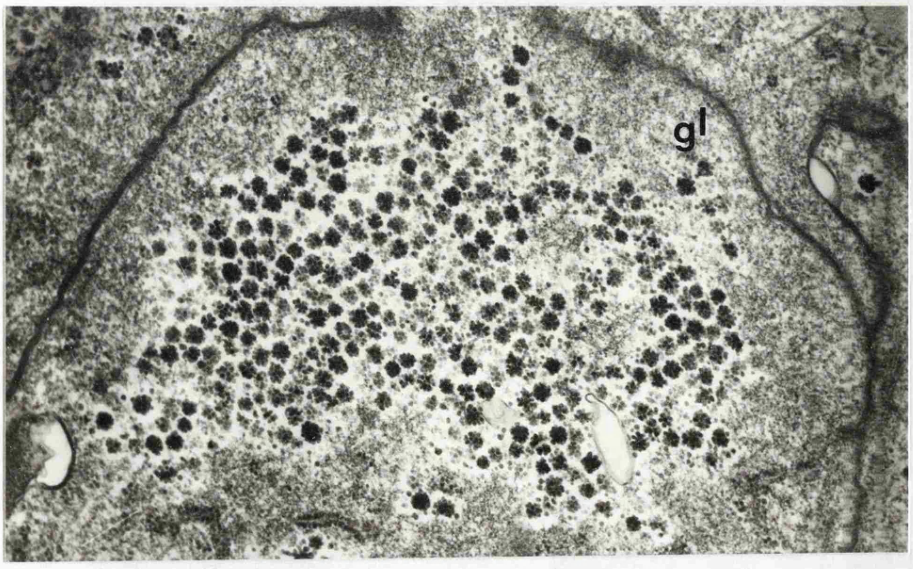


Plate 23. Enterocytes from the posterior midgut of a starved insect

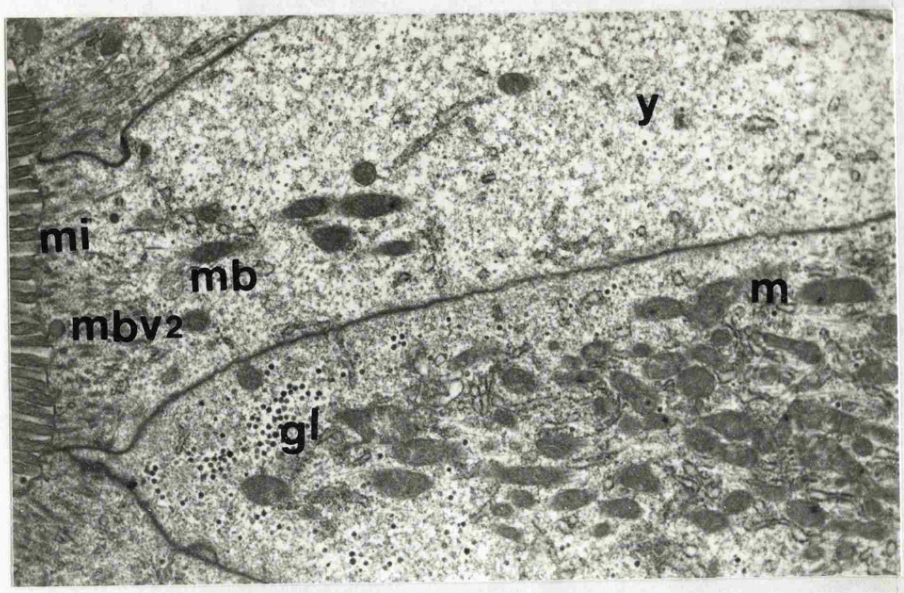
A. Apical region of the epithelium, a few membrane bound vesicles are in evidence. 'y' is substantially a "type 1" cell X10,171

B. "type 2" cells with a number of prominent Golgi bodies. X7,692

C. Similar to Plate 21C though not as many membrane bound vesicles X 12,786

mbv2, light coloured membrane bound vesicles; g, Golgi body; n, nucleus; mb, multivesicular; m, mitochondrion; gl, glycogen; mi, microvilli

A



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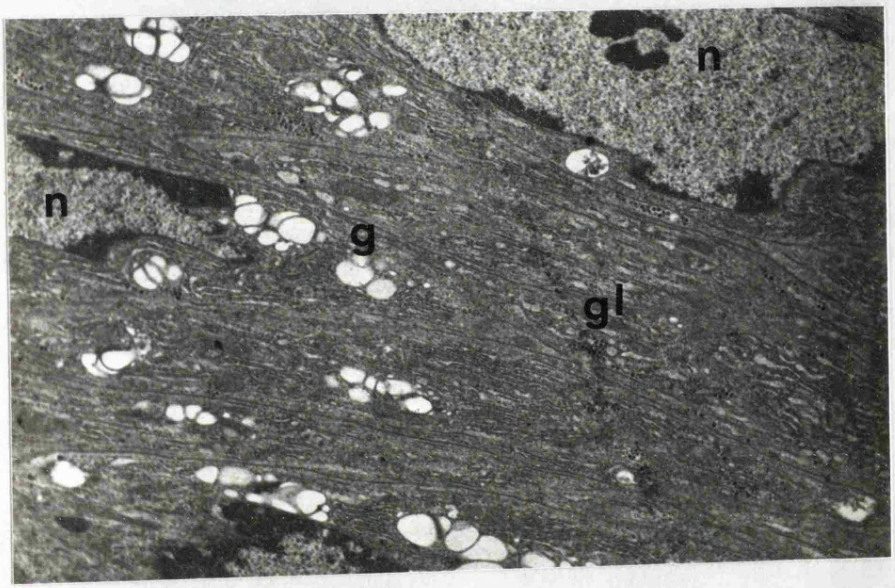


C

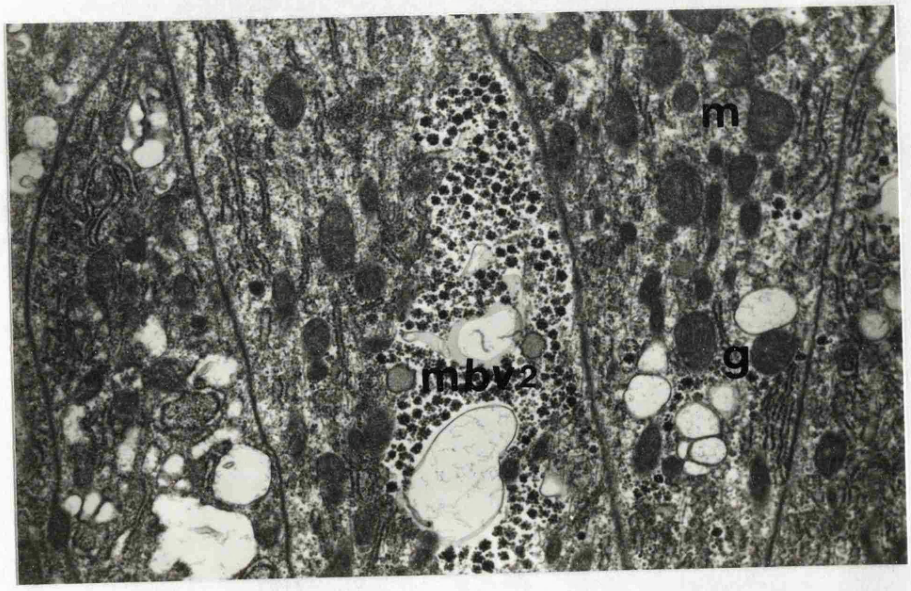


Plate 24. Scanning electron micrographs of the posterior midgut from a fed insect.

A. Surface view, note the regenerative nidi (ni) and tracheal tracks (t) X204

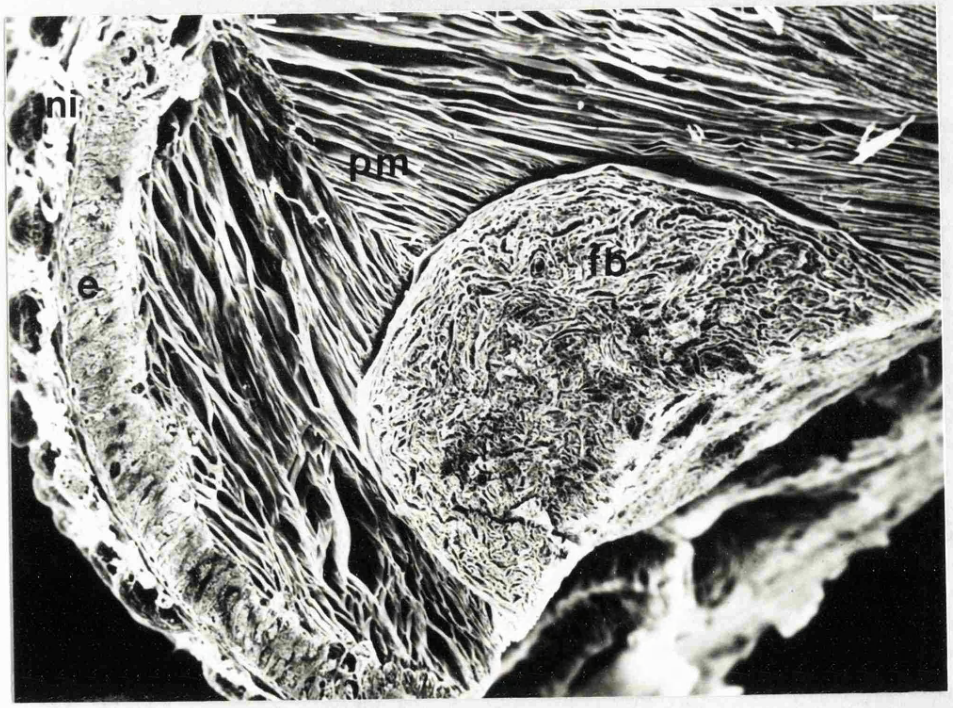
B. T.S. showing the concentric lamellae of the peritrophic membrane (pm) and the food bolus (fb) X 306

C. A slightly higher power micrograph illustrating a T.S. of a part of the midgut.

Note: ni, nidus; e, enterocytes; pm, peritrophic membrane X1020


C
t
ni
pm


ni
t
A


B
ni
pm
e
fb

Discussion

The general appearance of the midgut of young adult *Tenebrio molitor* noted in the present work is similar to that reported by Dadd (1954) and Gerber (1973). However, the superior definition of the light micrographs presented here, makes a detailed comparison of present and previous work impossible.

The apical membrane of the anterior and posterior, but not the middle, midgut was thrown into folds thus increasing the surface area for the release of digestive enzymes and the absorption of the products of digestion. In the fed insect the apical membrane was characterised by the presence of many cytoplasmic extrusions (ce). The interpretation of these structures has been the subject of controversy for many years. One school of thought has considered them to be related to secretory activity (Pradan, 1940), while the other concludes that they are the result of degenerative changes in the cytoplasm (Khan and Ford, 1962; Day and Powning, 1949). The consensus of opinion is in favour of the latter interpretation. Khan and Ford (1962) showed that in *Dysdercus fasciatus* ce appeared in response to starvation, and Day and Powning (1949) demonstrated that an increase in ce was not associated with an increase in enzyme concentration in the midgut contents of *Blatella germanica*.

In the present work, a more complete description of ce came from the ultrastructural study. They were found in a

number of shapes and sizes, some contained cellular organelles while others had little recognisable structure. Sometimes cytoplasmic extrusions were associated with type 1 cells (Plate 7) but not exclusively. The nucleus was never seen to accompany other organelles into a ce. In view of this, if ce were a form of secretion it should be termed 'merocrine' (nucleus-retained) rather than 'holocrine' (nucleus-lost) (Khan and Ford, 1962).

In contrast to the observations of Khan and Ford (1962) and Day and Powning (1949) (see above), cytoplasmic extrusions in young adult *Tenebrio molitor* were present in all regions of the guts of fed insects (see Chapter 3) and were conspicuously absent from starved insects. However, the variety of shape, size and content makes it seem unlikely that these structures are specifically designed for digestive enzyme release. Thus it would seem reasonable to conclude that they are indicative of cell breakdown associated with a high rate of enterocyte turnover in the fed animal. Following an ultrastructural study of the midgut of *Calliphora vicina* De Priester (1971) also concluded that apical cytoplasmic extrusions reflected a process of degeneration. However, elsewhere De Priester has indicated that extrusions may be at least in part an artefact of fixation (De Priester *et al.*, 1971).

In the present work the enterocytes of fed and starved insects exhibited a number of characteristics that might be related to processes of secretion and/or the absorption of the products of digestion:

1) Dilation of the cisternae of the rer.

2) Membrane bound vesicles. mbv were consistently found, they took one of two forms, viz. dense or light.

3) Microvesicles. Chains of mvl were found between the microvilli and contained a material that was similar in appearance to that in the swollen or vesiculated cisternae of the rer. mv2 formed at the tips of the microvilli (see Plate 10).

Since by definition starved insects will have no products of digestion to absorb, it seems likely that the structures described above are related to either peritrophic membrane (pm) formation and/or digestive enzyme secretion. The key question is which aspects of the morphology described above are related to pm secretion and which to enzyme secretion?

Richards and Richards (1971) pointed out that in general there is "... uncertainty as to the cell organelles involved in producing the material secreted for pm formation." They found "... no large or obvious change in organelles from the band of cells that secretes pm material to the more posterior ones which do not...". 13 years later the situation is still the same for those insects which produce type I membranes, despite Smith's (1968) seminal study on the type II pm of adult *C. vicina* (see introduction). In contrast ultrastructual studies have provided descriptions of the site of origin of the pm in the apical membrane of the midgut epithelium.

Tristram (1978) found that pm secretion in *Cionus scrophulariae* was restricted to the posterior midgut, where lightly staining strands of material and densely staining particles devolved from the microvilli and assembled in the midgut epithelium. Heinrich and Zebe (1973) reported strings of small vesicles between the microvilli in the midgut of larval *Locusta migratoria* which they suggested contributed to the formation of the pm in this insect. Similar structures (mvl) were present in the midgut of *Tenebrio molitor* and it seems reasonable to suggest that they serve a similar function. Once this assumption is made, then a possible interpretation of the cellular architecture can be made in terms of enzyme secretion and pm formation.

A gradient of α-glucosidase activity exists in the midgut such that anterior > middle > posterior (see Chapter 3 of the present work). In contrast Dadd (1954) found the reverse gradient for protease activity (anterior < posterior). Thus the overall enzyme secretory activity of the midgut may be uniform along its length. Production of the pm, however, appears to be greater at the anterior end of the midgut because 1. mvl are more prevalent anteriorly than posteriorly; 2) casual observations suggest that there is no substantial increase in the number of concentric lamellae in T.S.'s of the midgut from anterior to posterior. From the above it may be concluded that any additional differences in cellular architecture between the two ends of the midgut epithelium are likely to be associated with pm formation, whilst similarities of structure

may be associated with enzyme secretion.

Type 1 cells and cells with type 1 regions close to the apical membrane are more prevalent in the anterior midgut than elsewhere and it is interesting to note that Richards (1975) found an organelle-free region at the base of the microvilli in the midgut of mosquito larvae in the region where pm is secreted. However, mvl are more prevalent in type 3 cells than in type 1 cells. The former cell type are less numerous in fed posterior and starved midguts, which also seem to produce little pm. Therefore the characteristic features of type 3 cells, namely swollen or vesiculated cisternae of the rer, may be involved with the production of pm material. In which case the following sequence may be proposed: pm material produced in the rer and released in strings of small vesicles (mvl) between the microvilli. In line with this proposal Lehane (1976) suggested that transportation of digestive enzymes in vesicles derived from the rer and released from the microvilli might be a general secretory mechanism in insect midgut cells. His comments were aimed at solving the problem of how to explain (enzyme) secretion in most insect guts where rer and Golgi apparatus are prominent but there is a dearth of mbv; a similar problem exists for the production of pm material (see above). In support of his hypothesis Lehane (1976) pointed out that vesiculation of the microvilli has been described in the midgut of cells of *Dacus oleae* (Baccetti, 1962), *Calliphora vicina* (De Priester, 1971), *Sarcophaga*

bullata (Nopanitaya and Misch, 1974), *Stomxys calcitrans* (Lehane, 1976), in the latter two species the microvesicles have a similar diameter to those of vesicles budded from the rer.

Some vertebrate secretory cells shorten the " **exocrine** pancreas secretory sequence". Specifically, fibroblasts, chondryocytes and plasma cells omit the concentration step and thus fail to form mbv in Golgi bodies; the cells store the product for only a short time, or not at all, and discharge the product continuously (Palade, 1975). Lehane's (1976) scheme for secretion in the insect midgut is consistent with Palade's description, as is the fact that for most insects digestive enzyme activity is principally luminal; there is little stored in epithelial tissue (see Chapter 3).

In contrast to many insects mbv are a consistent feature of all regions of the *Tenebrio* midgut. In terms of the pre-suppositions on page 168, the ubiquity of mbv2 denotes their possible relationship to enzyme secretion. mbv2 are particularly prevalent close to the apical membrane and are associated with Golgi bodies. However, fusion of mbv2 with the apical plasma membrane and discharge of their contents (eccrine secretion) has not been observed, thus it is still uncertain whether these vesicles are involved with secretion. mbv2 are not as numerous in the *Tenebrio* midgut as comparable structures are in the opaque cells of *Stomxys calcitrans* (Lehane, 1976). This does not preclude their involvement in

enzyme secretion in *Tenebrio*. *Stomxys* is an intermittent feeder and as a result enzyme secretion occurs in large amounts over relatively short periods. *Tenebrio*, on the other hand, is more or less a continous feeder (see Chapter 3) whose secretory activity will be less extreme.

The occurrence of glycogen rosettes in the posterior midgut may be seen as evidence for energy dependent processes within the enterocytes n.b. Jamieson and Palade (1968) related glycogen deposits in vertebrate exocrine pancreatic cells to an energy-dependent "lock" between the rer and the Golgi bodies. The fact that glycogen in the *Tenebrio* midgut was present in fed and starved insects argues against it being derived *en passant* from the absorbed products of digestion.

A potential indictment against a role for mbv2 in enzyme secretion is the fact that they are less abundant in starved midguts than in fed, yet both treatments exhibit a 6-fold increase in the titre of α-glucosidase in the first 4 days of adult life (see Chapter 3). The latter observation suggests that the level of enzyme production is comparable in the two treatments, consequently one would expect to find the organelles responsible to be present to the same extent in starved and fed insects. However, the situation is more complicated than would appear at first sight. The enzyme titre in the starved insect reached a peak on the 4th day whereas in the fed insect the maximum activity was not attained until 7 days after the last

ecdysis (see Chapter 3). Abboud (1981) found that ablation of the pars intercerebralis affected the α-glucosidase activity in the midgut tissue before that of the contents. Thus it may be expected that for any given treatment the maximum total midgut α glucosidase activity would occur after the machinery responsible for its production has gone into decline. If so, it would be expected that in the case of a 4-day starved insect, when total enzyme activity was maximal, cellular organelles involved in enzyme synthesis and release would be less evident. Conversely, 4-day old fed insects would be actively synthesizing and releasing enzyme, and the relevant organelles would be prominent.

An additional argument for expecting a difference in the rate of enzyme synthesis and hence in the extent of the machinery is the fact that in a fed insect the turnover of enzyme molecules in the lumen of a fed insect is bound to be greater than in the starved insect because of the movement of food through the gut.

Three cell "types" have been described in the midgut of *Tenebrio* in the present work. However, one cell can exhibit areas which have the features of all 3 cell "types" suggesting that the descriptions are not mutually exclusive and that they do not represent morphologically distinct populations of cells. Since no serial sections were examined it is not known whether most cells exhibit areas characteristic of all 3 descriptions. If it is assumed, however, that there are cells which are pre-

dominantly of one "type", alongside others which are "chimeric" then an alternative interpretation is that an enterocyte changes in form during the course of its existence. Again the present work does not provide any evidence for or against this hypothesis.

On the basis of sequential histological observations Gander (1968) suggested that the mosquito midgut may exhibit a temporal separation of synthetic activities i.e. produce pm precursors in the first half of its functional life and digestive enzymes in the second. Richards and Richards (1977) concluded that such a temporal separation is not found in insects (like *Tenebrio*), that under normal circumstances produce pm continuously. Certainly mbv2, swollen rer and strings of microvesicles (mvl) were found in the same *Tenebrio midgut* cell (Plate 10).

Summary

3 "types" of enterocyte have been described in the midgut of adult *Tenebrio molitor*. It is suggested that these "cell types" may simply reflect different stages in the ageing of a single population of cells.

The ultrastructure of the enterocytes is discussed in terms of a dual function of enzyme secretion and production of the peritrophic membrane. It is suggested that pm precursors are manufactured in distended cisternae of the rer and released into the lumen as strings of small vesicles. The fine structure of both starved and fed midguts is consistent with the hypothesis

that digestive enzymes are manufactured in the rer, packaged by the Golgi body into membrane bound vesicles (mbv2) which are then released into the lumen; though release of the contents of mbv into the lumen has not yet been observed. Cytoplasmic extrusions are only found in fed insects and it is concluded that these are the result of cell breakdown associated with a high rate of enterocyte turnover and are not related to enzyme secretion.

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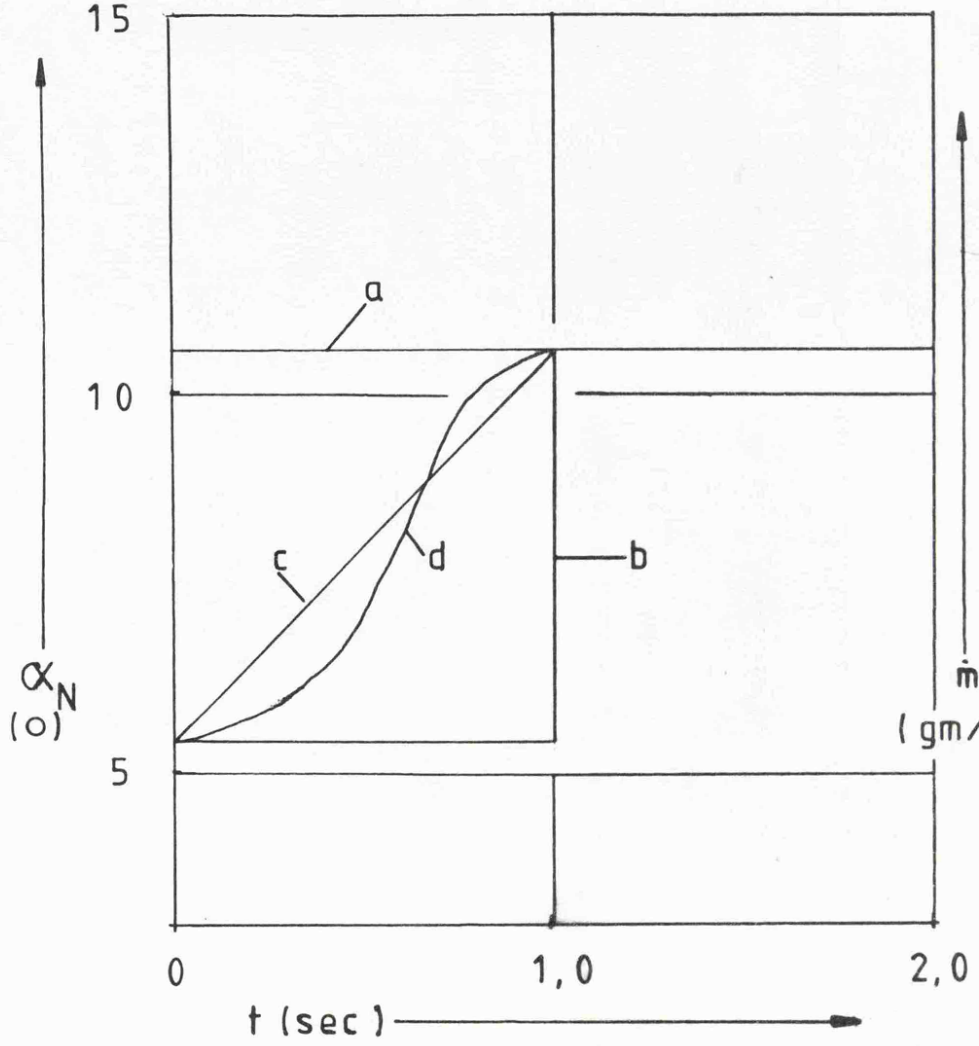
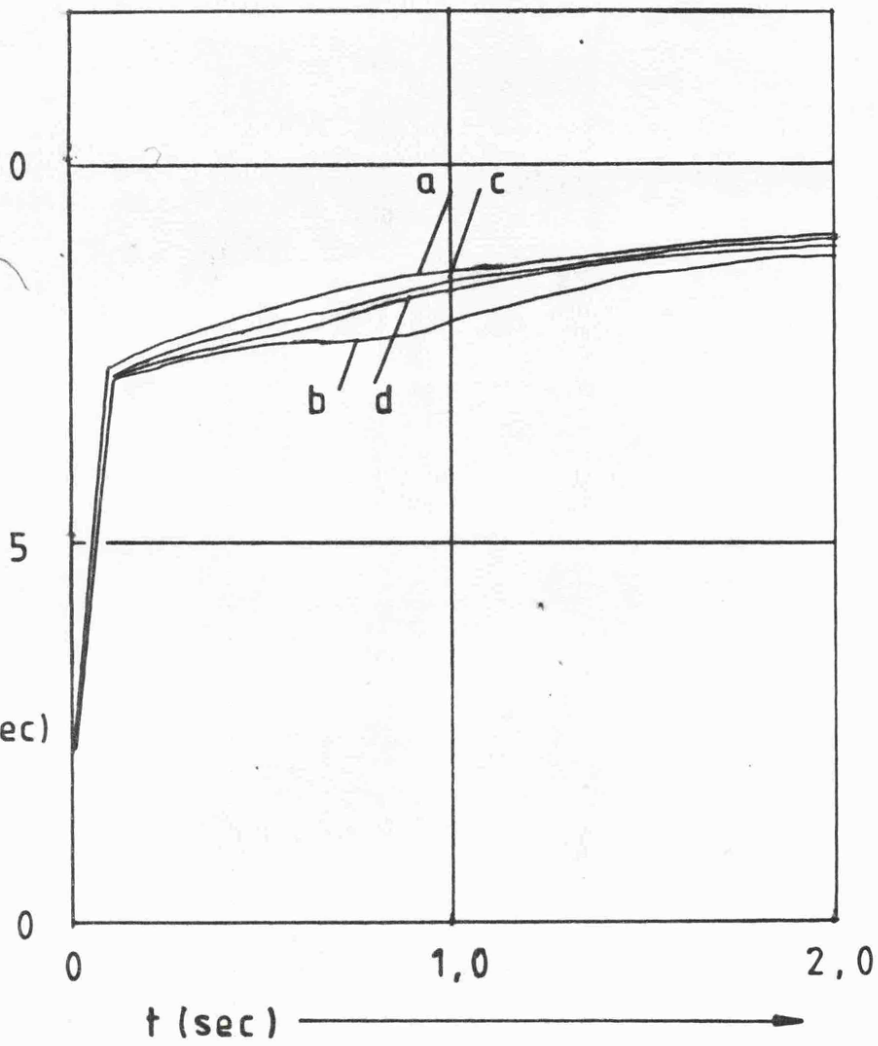
FIG(6,1) α_N VS TIME



FIG(6,2) $\dot{m}f$ VS TIME



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BURSICON IN THE MEALWORM, *TENEBRIO MOLITOR* L. AND ITS ROLE IN THE CONTROL OF POSTECDYSIAL TANNING

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Abstract—Using the adult *Calliphora* bioassay, we found that the tanning hormone, bursicon, is present in the blood of pupal and adult *Tenebrio* only at the time of ecdysis, when it is released massively from the thoracic and abdominal central nervous system. The hormone's half life in the blood is short (about 1–2 h). Contrary to the findings of other workers, we could find no evidence for the presence of the hormone in the haemolymph during pharate adult development, before ecdysis begins. When newly ecdysed pupae were ligated about the neck, adult development of the thorax and abdomen proceeded normally, but postecdysial tanning of the adult cuticle was almost completely prevented. This failure to tan was not due to lack of bursicon as the hormone was released normally in the ligated animals at the time of ecdysis. This suggests that a pre-ecdysial signal may be required for the development of epidermal competence to respond to bursicon.

Key Word Index: Mealworm, *Tenebrio*, hormones, bursicon, cuticle, tanning, ecdysis

INTRODUCTION

Rapid postecdysial tanning of the cuticle has been shown to be controlled by the peptide hormone bursicon in most insects which have been studied (Reynolds, 1983). The hormone is released massively into the blood during or immediately after ecdysis and is thereafter rapidly eliminated. It is conspicuously absent from the blood at other times.

An apparent exception to this general pattern is the beetle, *Tenebrio*. In a series of papers, Delachambre and his colleagues (Delachambre, 1971; Grillot *et al.*, 1976; Delachambre *et al.*, 1979a; 1979b) have proposed a very different model for the hormonal control of postecdysial tanning in adult mealworms, in which a tanning hormone ("bursicon") is released *before* ecdysis. Moreover, this hormone is supposed not to be released in a single pulse, but is present in the blood for a period of about 2 days.

This model is based on two lines of evidence. In ligature experiments, Delachambre (1971) showed that normal tanning of the thoracic and abdominal sternites was prevented by either neck or thoracic/abdominal ligatures placed prior to the fourth day of pupal life. However, after day 6, tanning could not be prevented. This critical period implied the release of a tanning hormone well before ecdysis (which occurred on day 9). Evidence supporting this idea was obtained from measurements of "bursicon" in the blood and in the central nervous system. A conspecific bioassay was used in which isolated abdomens received injections of blood or tissue extracts at about the time of adult ecdysis. The tanning factor was present principally in the thoracic and abdominal ganglia of the ventral nerve cord and their associated neurohaemal organs (perivisceral organs), from which it was apparently released at about the time of the critical period (Grillot *et al.*, 1976). The tanning factor was detectable in the blood from this time, rising gradually to a peak titre at the time of ecdysis (Delachambre *et al.*, 1979a).

This scheme for the control of tanning in *Tenebrio* appeared so completely at variance with what is known for other insects that we resolved to reinvestigate the problem using the classical adult blowfly bioassay for bursicon devised by Fraenkel and Hsiao (1965).

MATERIALS AND METHODS

Mealworms, *Tenebrio molitor* L, were purchased from suppliers as mature larvae. They were fed on bran and carrots and freshly moulted pupae were collected daily. The insects were kept at 25°C, approx. 50% r.h. under a 12 h light, 12 h dark photoperiodic regime. Adult development was staged as days after pupation, except for pharate adults nearing the time of ecdysis. In this case we established that the onset of moulting fluid resorption (first visible on the legs) corresponded to a time about 10 h before ecdysis. This method was used in taking tissue samples from insects, as in Fig. 4. Where blood samples were taken from pharate adults which were closer to ecdysis than this (as in Figs 1 and 5), then the time at which the sampled insect subsequently began ecdysial behaviour was noted.

Larvae of the blowfly, *Calliphora vomitoria* Meigen, were obtained from a local fishing-bait supplier and allowed to pupate in sawdust. In order to

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synchronise adult eclosion, the pharate adults were kept from 1 to 7 days in the refrigerator (approximately 4°C) following the emergence of the first few flies in each "batch". On return to room temperature, most of the remaining flies eclosed within the first few minutes.

Blood samples were taken from *Tenebrio* by amputating a metathoracic leg; in some cases very gentle pressure was applied to facilitate bleeding. The haemolymph was collected in 5 μl Drummond microcaps and stored at −20°C until required. For tissue samples, insects were dissected under Ephrussi and Beadle's saline, the central nervous system removed, then briefly blotted and stored frozen. When needed for bioassay, pools of 20 tissues were suspended in 160 μl saline solution, sonicated for 2 min (MSE Soniprep 150) and centrifuged briefly to remove cellular debris (Eppendorf 5412 micro centrifuge).

Bursicon was detected by bioassay on neck-ligated adult blowflies, using essentially the procedure described by Fraenkel and Hsiao (1965). Blowflies were neck-ligated without anaesthetic as they emerged from their puparia, ensuring that the ptilinum was filled with blood. The ligature prevents the release of bursicon and thus post-ecdysial tanning. The test insects were left for 2–3 h at room temperature prior to injection so that the small proportion of flies which tanned (due to poor ligatures) could be discarded. Injections (2 μl) of blood or tissue extracts were made into the scutellum of ether-anaesthetised flies using a calibrated glass needle attached to a mouthpiece by a rubber tube. Flies treated in this way did not bleed after injection and so we did not attempt to seal the wound at the injection site. The injected flies were kept for 3 h at room temperature and then immersed in 70% ethanol to terminate the assay and render cuticular colouration more easily visible. Tanning of the thoracic and abdominal sclerites was assessed soon after the termination of the assay using a four-point scoring system similar to that used by Vincent (1972). In order to quantify bursicon, blood and tissue samples were appropriately diluted so that the tanning response obtained in the assay was on the linear portion of the dose–response curve. Bursicon activity was then computed as a "relative score", being the actual score achieved in the bioassay multiplied by the dilution of the sample.

Post-ecdysial tanning in *Tenebrio* was assessed by a method similar to that used by Delachambre (1971). The insects were examined 48 h after ecdysis and assigned a score from 0–4 according to the extent of tanning of the abdominal sternites, as follows: 0–untanned; 1–mostly untanned with light brown patches; 2–mostly light brown with untanned patches; 3–uniformly light brown; 4–uniformly dark brown.

RESULTS

In preliminary experiments, Fraenkel and Hsiao (1965) established that the blood of ecdysing adult mealworms contained a factor which was active in the blowfly bioassay for bursicon. We chose this rapid and simple method to quantify the hormone in both blood and tissues of *Tenebrio*, because we entertained considerable doubts about the validity of the conspecific bioassay of Delachambre as a measure of bursicon (see Discussion).

Table 1. The haemolymph titre of bursicon in pupae and pharate adults of *Tenebrio molitor*

Days after pupal ecdysis	Relative score Mean	SE	N
0*	24.8	2.1	10
1	0.15	0.12	10
2	0.05	0.05	9
3	0.05	0.05	9
4	0	0	10
5	0.06	0.06	8
6	0.06	0.05	9
7	0.13	0.08	8
8	0.10	0.10	7
9*	33.0	0.5	11
Control	0.07	0.07	7

*Sampled 30 min after the start of pupal ecdysis (day 0) or adult ecdysis (day 9). These gave mean values which were significantly different (t-test, $P < 0.001$) from the control (flies injected with 2 μl saline alone). Other samples gave mean values which were not significantly different from the control ($P > 0.6$).

First we established that bursicon is present in the blood only at the time of ecdysis (see Table 1). Whereas considerable amounts of the hormone could be detected in the blood of ecdysing pupae and adults using the blowfly bioassay, the extent of tanning in flies which received blood from intermoult pupae and pharate adults was not significantly different from that produced by injections of saline alone.

We found that release of the hormone was a brief event. Maximal titres were achieved in insects sampled 30 min after the start of ecdysis. The hormone subsequently disappeared rapidly from the blood,

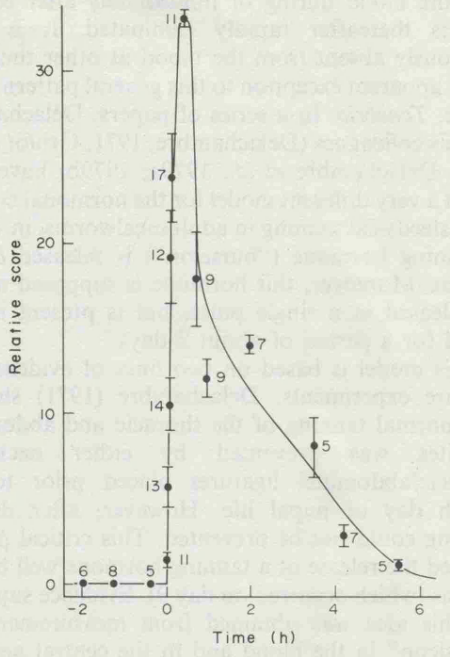

Fig. 1. Bursicon activity in the blood of *Tenebrio molitor* during adult acdysis. Abscissa: time in h after the start of ecdysis. Ordinate: relative score (=actual bioassay score multiplied by dilution of sample). Results are means (±SE) of the number of determinations indicated. The continuous line is fitted by eye, and is drawn assuming that release of hormone begins at the start of ecdysis (see text).

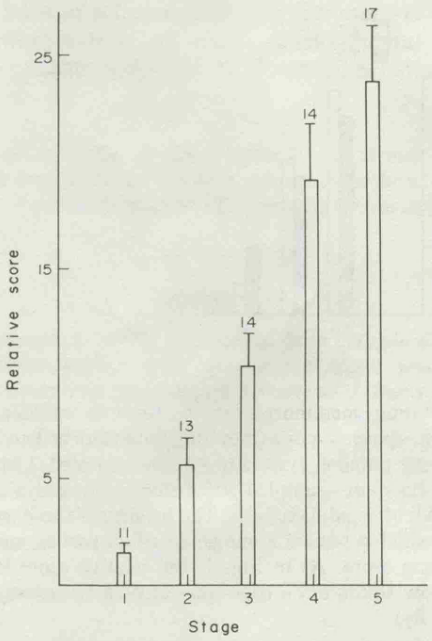

Fig. 2. Correlation between blood titres of bursicon and behaviour during adult ecdysis in *Tenebrio molitor*. Abscissa: stage 1—rolling behaviour; stage 2—abdomen straightens (early peristalsis); stage 3—backward movement of old cuticle (late peristalsis); stage 4—splitting of old pronotal cuticle; stage 5—exuvia shed. Stages 1–4 take about 2 min, stages 4–5 take about 12 min. Other details as for Fig. 1.

with an approximate half life of 1–2 h. The extreme rapidity of bursicon release was established by taking blood samples from individuals at closely observed stages of ecdysis (see Fig. 2). A small amount of hormonal activity was detectable in the blood of insects which were bled at the first sign of ecdysial behaviour. Since it took a few seconds to bleed the insects, we interpret this to mean that the release of bursicon begins at the same time as overt ecdysial behaviour. We never found bursicon activity in pharate adults which had completed resorption of moulting fluid but which had not yet begun ecdysis. By the time the old cuticle was split, only 2 min after the beginning of ecdysis, the titre of bursicon in the blood had already reached 60% of the maximal value that we recorded.

The pattern of release at pupal ecdysis was similar in every respect, although the maximal titre achieved was somewhat lower than at adult ecdysis (Fig. 3).

All parts of the central nervous system examined were found to contain some bursicon activity detectable by the blowfly bioassay. However, more than 90% of the total activity was present in the thoracic and abdominal ganglia of the ventral nerve cord. Titres of hormone in these tissues declined markedly during both pupal and adult ecdysis, presumably denoting its release into the blood (Fig. 4). By contrast, the titres measured in the central nervous system during pupal and pharate adult development remained constant.

The amounts of bursicon which disappear from the central nervous system during pupal and adult ecdysis are insufficient to account for all the hormone which appears in the blood. It is possible that this is due to the fact that we deliberately excluded the perivisceral organs from our nervous system samples, because it is difficult to ensure that they are dissected away from surrounding tissues in a uniform way. However, when we did attempt to assay thoracic and abdominal ganglia together with their perivisceral organs (data not shown) we did not greatly increase the amount of bursicon activity. Thus it is possible that the hormone is modified in some way as it secreted, so as to increase its bioactivity.

The above findings do not support Delachambre's suggestion that in *Tenebrio* "bursicon" is released considerably before adult ecdysis. We sought to explain the discrepancy between our findings and his by making the hypothesis that Delachambre's critical period on days 4–6 represents not the release of bursicon itself, but another, previous endocrine event which is required for bursicon's release. We tested this hypothesis by measuring bursicon titres during ecdysis in insects which had been neck-ligated 1–3 h after pupal ecdysis. The ligated insects showed very little of the cuticular tanning which normally occurs after adult ecdysis, even though their pre-ecdysial tanning was similar to that seen in unligated insects. The tanning score achieved by the ligated animals was 0.8 ± 0.1 ($N = 54$), whereas unligated insects normally show a uniform score of 4.0. This finding is similar to the results of Delachambre (1971). It is clear from Fig. 5, however, that bursicon release occurs normally in these animals. The time course of bursicon activity in the blood of ecdysing ligated adults (as measured by the blowfly bioassay) was almost identical to that measured in intact insects (Fig. 1.)

DISCUSSION

The results of this study are consistent with findings in other insects (reviewed by Reynolds, 1983), in that in *Tenebrio* bursicon is released into the

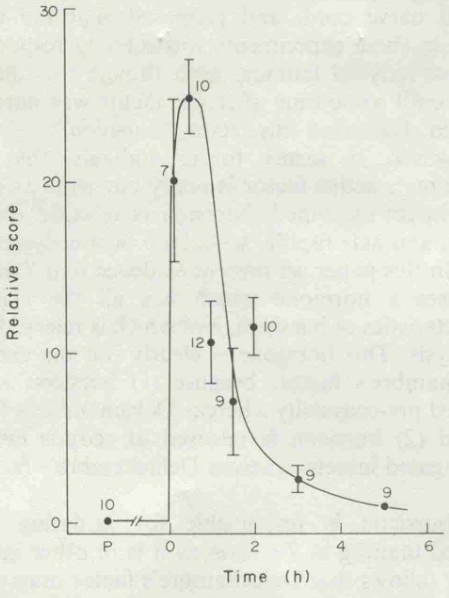

Fig. 3. Bursicon activity in the blood of *Tenebrio molitor* during pupal ecdysis. P—pharate pupa (moulting fluid resorbed and therefore within a few hours of ecdysis). Other details as for Fig. 1.

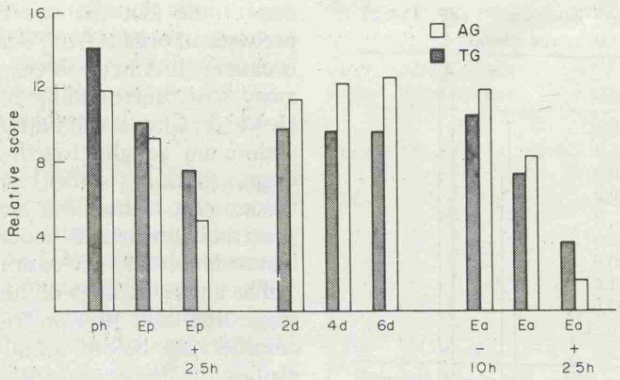

Fig. 4. Titres of bursicon in the ventral nerve cord during metamorphosis in *Tenebrio molitor*. TG—thoracic ganglia; AG—abdominal ganglia. Abscissa: ph—pharate pupa (moulting fluid resorbed and therefore within a few hours of ecdysis); Ep—sampled during pupal ecdysis; Ep + 2.5 h—sampled 2.5 h after pupal ecdysis; 2 d, 4 d, 6 d—days after pupal ecdysis; Ea-10 th—sampled 10 h before adult ecdysis; Ea—sampled during adult ecdysis; Ea + 2.5 h—sampled 2.5 h after adult ecdysis. The heights of the bars indicate the means of two separate experiments, in each of which a pooled homogenate of 20 tissues was bioassayed by injection into 10 flies to give a mean tanning score. As in Fig. 1, the relative score is computed as the actual tanning score achieved multiplied by the dilution (one quarter of a tissue was injected into each fly).

blood only at the time of ecdysis in the form of a brief pulse. In particular, we found no evidence for pre-ecdysial release of bursicon in *Tenebrio* as has been suggested to occur by Delachambre and his co-workers.

In agreement with Delachambre (1971) we found that neck ligation 1–3 h after pupal ecdysis almost completely prevented post-ecdysial tanning. Delachambre showed that this failure to tan following otherwise normal adult development was due to the absence from the blood of a factor normally released from the perivisceral organs of the ventral nerve cord about 2 d before adult ecdysis. Evidently this release required some unknown activating signal from the head. He was able to restore tanning in the ligated insects by injecting active blood, or extracts of the ventral nerve cord, and proposed that the active factor in these experiments was directly responsible for post-ecdysial tanning, even though this did not occur until some time after the factor was normally released. He called this factor "bursicon".

However, it seems to us unlikely that Delachambre's active factor is really bursicon. In every other insect examined, bursicon is released only at ecdysis and acts rapidly to initiate post-ecdysial tanning. In this paper we present evidence that *Tenebrio* possesses a hormone which has all the classical characteristics of bursicon, and which is released only at ecdysis. This hormone is clearly not the same as Delachambre's factor, because (1) bursicon is not released pre-ecdysially whereas Delachambre's factor is, and (2) bursicon is released at ecdysis even in neck-ligated insects, whereas Delachambre's factor is not.

If bursicon is responsible for initiating post-ecdysial tanning in *Tenebrio* as it is in other insects, then it follows that Delachambre's factor must affect tanning in some other way. Since we have shown here that bursicon is released normally even in neck-ligated insects which do not tan, then Delachambre's factor cannot act to promote or permit the release of bursicon. We suggest that Delachambre's factor may act to promote the competence of the epidermis to respond to bursicon when this is subsequently released at the time of ecdysis. Just such an acquisition of competence occurs in *Manduca*, where the integument of the pharate-adult moth is unresponsive to bursicon until a few hours before the hormone is released (Reynolds *et al.*, 1979). The proximate cause of the acquisition of bursicon responsiveness in *Manduca* is not known, although it may depend on the fall in the titre of haemolymph ecdysteroids which occurs before ecdysis, and which appears to be essential for

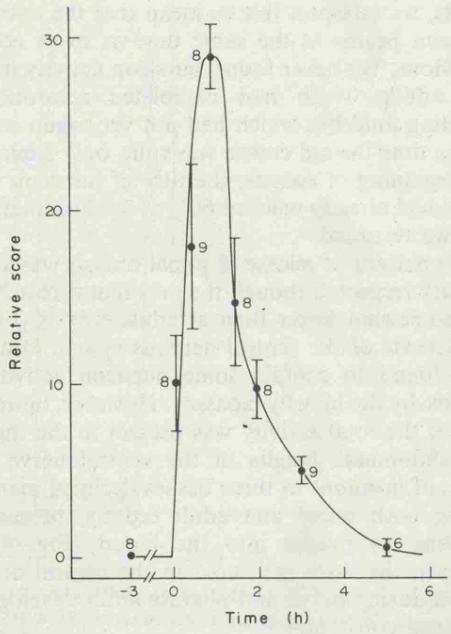

Fig. 5. Effect of neck ligation on bursicon activity in the blood of *Tenebrio molitor* during adult ecdysis. Neck ligation was performed 1–3 h after pupal ecdysis. Details as for Fig. 1.

the release of eclosion hormone (Truman, 1981) and the coordination of other developmental events which precede ecdysis (J. W. Truman, personal communication).

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